

Animal Science, Issues and Professions

ANIMAL SCIENCE AND ISSUES

Jacob M. Jaworski
Editor

NOVA

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

ANIMAL SCIENCE AND ISSUES

No part of this digital document may be reproduced, stored in a retrieval system or transmitted in any form or by any means. The publisher has taken reasonable care in the preparation of this digital document, but makes no expressed or implied warranty of any kind and assumes no responsibility for any errors or omissions. No liability is assumed for incidental or consequential damages in connection with or arising out of information contained herein. This digital document is sold with the clear understanding that the publisher is not engaged in rendering legal, medical or any other professional services.

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

Additional books in this series can be found on Nova's website
under the Series tab.

Additional E-books in this series can be found on Nova's website
under the E-book tab.

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

ANIMAL SCIENCE AND ISSUES

JACOB M. JAWORSKI
EDITOR



Nova Science Publishers, Inc.
New York

Copyright © 2011 by Nova Science Publishers, Inc.

All rights reserved. No part of this book may be reproduced, stored in a retrieval system or transmitted in any form or by any means: electronic, electrostatic, magnetic, tape, mechanical photocopying, recording or otherwise without the written permission of the Publisher.

For permission to use material from this book please contact us:

Telephone 631-231-7269; Fax 631-231-8175

Web Site: <http://www.novapublishers.com>

NOTICE TO THE READER

The Publisher has taken reasonable care in the preparation of this book, but makes no expressed or implied warranty of any kind and assumes no responsibility for any errors or omissions. No liability is assumed for incidental or consequential damages in connection with or arising out of information contained in this book. The Publisher shall not be liable for any special, consequential, or exemplary damages resulting, in whole or in part, from the readers' use of, or reliance upon, this material. Any parts of this book based on government reports are so indicated and copyright is claimed for those parts to the extent applicable to compilations of such works.

Independent verification should be sought for any data, advice or recommendations contained in this book. In addition, no responsibility is assumed by the publisher for any injury and/or damage to persons or property arising from any methods, products, instructions, ideas or otherwise contained in this publication.

This publication is designed to provide accurate and authoritative information with regard to the subject matter covered herein. It is sold with the clear understanding that the Publisher is not engaged in rendering legal or any other professional services. If legal or any other expert assistance is required, the services of a competent person should be sought. FROM A DECLARATION OF PARTICIPANTS JOINTLY ADOPTED BY A COMMITTEE OF THE AMERICAN BAR ASSOCIATION AND A COMMITTEE OF PUBLISHERS.

Additional color graphics may be available in the e-book version of this book.

LIBRARY OF CONGRESS CATALOGING-IN-PUBLICATION DATA

Animal science and issues / editors, Jacob M. Jaworski.

p. cm.

Includes index.

ISBN 978-1-61122-124-4 (eBook)

1. Zoology--Miscellanea. I. Jaworski, Jacob M.

QL81.A53 2010

590--dc22

2010033041

Published by Nova Science Publishers, Inc. † New York

CONTENTS

Preface	ix
Population Dynamics and Secondary Production of the Snail <i>Neritina Virginea</i> (Linnaeus, 1758) (Mollusca: Gastropoda: Neritidae) in an Estuary in Southern Brazil <i>Ricardo S. Absalão, Ricardo S. Cardoso and Alexandre S. Alencar</i>	1
Reproduction and Life History in the Two Land Snails <i>Monacha</i> <i>Cartusiana</i> (Müller) and <i>Eobania Vermiculata</i> (Müller) (Helicidae: Mollusca) in the Laboratory <i>M. I. Mohamed and R. F. Ali</i>	17
Effect of Sublethal Concentration of Arsenic on Hemocyte Density in Edible Crab, <i>Scylla serrata</i> <i>Sanjib Saha, Mitali Ray and Sajal Ray</i>	27
Immunotoxicity of Azadirachtin in Freshwater Mussel in Relation to Surface Adhesion of Hemocytes and Phagocytosis <i>Suman Mukherjee, Mitali Ray and Sajal Ray</i>	37
Aggregation and Chemical Induced Interference of Aggregation of Hemocytes of Indian Mudcrab Exposed to Arsenic <i>Sajal Ray, Mitali Ray and Sanjib Saha</i>	45
Developmental Expression Profile of Cockroach Vitellogenin Genes Vg 1 and 2 <i>Azza Mohamed Elgendy, Mohamed Elmogy, Muhammed Tufail and</i> <i>Makio Takeda</i>	57
Lectin Binding Pattern of Intestinal Goblet Cells in Horse, Pig and Rabbit <i>J. M. Galotta, S. G. Márquez, C. N. Zanuzzi, E. J. Gimeno, E. L.</i> <i>Portiansky and C. G. Barbeito</i>	67

Ultrastructural Aspects and Frequency of Ciliated Fibroblasts in the Periodontal Ligament of Incisor Tooth of Rats Submitted to Different Eruption Conditions	77
<i>Pedro Duarte Novaes, Simone de Cássia Barbosa, Nádia Fayez Omar, Juliana dos Santos Neves, Eliene Aparecida Orsini Narvaes, Maria Albertina de Miranda Soares and José Rosa Gomes</i>	
Zooplankton Composition in an Estuarine Area of the State of Maranhão, Northeastern Brazil	87
<i>Sérgio Luiz Costa Bonecker and Cristina de Oliveira Dias</i>	
Morphology and Distribution of Four Pill Millipedes (<i>Arthrosphaera</i>) of the Western Ghats	99
<i>B. S. Kadamannaya and K. R. Sridhar</i>	
Comparison of Neighbor-Joining and Maximum-Parsimony Methods for Molecular Phylogeny of <i>Oryx</i> Species Using 12S rRNA and 16S rRNA Gene Sequences	117
<i>Ibrahim A. Arif, Haseeb A. Khan, Ali H. Bahkali, Ali A. Al Homaidan, Ahmad H. Al Farhan, Mohammad Shobrak and Mohammad Al Sadoon</i>	
Haematological Parameters in Adult <i>Channa Punctatus</i> (Bloch) Subjected to Multistress with an Organophosphate Pesticide Coupled with Challenge of Aeromonads	127
<i>Rajarshi Ghosh and Sumit Homechaudhuri</i>	
Description of Two New Species of Mites Infesting Edible Mushroom (<i>Pleurotus Ostreatus</i>)	141
<i>Amrita Mukherjee, S. K. Gupta and G. K. Saha</i>	
Usefulness of Noninvasive Methods of DNA Sampling but with a Caution	151
<i>Ibrahim A. Arif, Haseeb A. Khan, Ali A. Al Homaidan, Ahmad H. Al Farhan, Ali H. Bahkali, Mohammad Shobrak and Mohammad Al Sadoon</i>	
Thyroid Hormone Mediated Maintenance of Glucose Homeostasis and Cytomorphology of Cardiac Muscle and Skin in Rat	157
<i>Sudipta Chakrabarti, Srikanta Guria, Nisha Balmiki and Madhusudan Das</i>	
Oncotic Cytology in the Prevention of Cervical Cancer	165
<i>Melissa Cesar, Luma Rebeca Pedroso, Marcela Cristina Altvater, Liya Mikami and Kárita Cláudia Freitas-Lidani</i>	
Cytotoxic Response and Nonspecific Phagocytosis as Innate Immunity of Mollusc under Arsenic Exposure	173
<i>Sudipta Chakraborty, Mitali Ray and Sajal Ray</i>	

Spawning of the Endangered Killifish, <i>Aphanius Disar Dispar</i> (Rüppel, 1829), under Laboratory Conditions <i>El Amin Mohamed Suliman, Ali S. Al-Akel, Hmoud F. Alkhem Al-Balawi, Fahad Al-Misned and Zubair Ahmad</i>	185
Structure of a Glucan and a Heteropolymer from the Cell Wall of the Yeast <i>Phaffia Rhodozyma</i> <i>Juliana Negrão Borges, Tania Maria Bordin Bonfim, Guilherme Lanzi Sassaki, Ricardo Wagner, José Domingos Fontana, Sandra Mara Woranovicz-Barreira</i>	193
Primary Immune Response and Parasite Dissemination in Canine Visceral Leishmaniasis <i>P. S. T. Veras, W. L. C.dos-Santos, A. T. F.lmeida and C. A. T. Rodrigues</i>	205
Abstracts Presented in - IV International Symposium on Developmental Biology and III Student Meeting on Developmental Biology <i>Chao Yun Irene Yan</i>	223
Abstracts Presented in - X Brazilian Symposium on Extracellular Matrix and V International Symposium on Extracellular Matrix <i>Marimellia Porcionato</i>	271
Index	439

PREFACE

This book presents a comprehensive review of various studies in animal science. Topics discussed herein include the developmental expression profile of cockroach genes; zooplankton composition in an estuarine area; noninvasive methods of DNA sampling; morphology and distribution of four pill millipedes; and immunotoxicity of azadirachtin in freshwater mussle.

Chapter 1 - The population of *Neritina virginea* (Linnaeus, 1758) in Barra Grande estuary, Ilha Grande, state of Rio de Janeiro in southern Brazil (23° S) was observed from February 1999 through December 2000. The sandflat where the neritids live was divided into 5 sectors, each 30 m long, and each sector was sampled separately. Densities were higher in spring and lower in winter. Reproduction was continuous year-round, but with increased activity in early spring. Fecundity was about 80 eggs/embryos per capsule, and the sex-ratio was 1 males: 2.89 females. This population of *N. virginea* attained higher densities and shell size in the inner part of the estuary. A total of 40,596 individuals was sampled, with shell length varying between 2.50 and 18.80 mm. Life span varied between 1.78 and 2 years for the first and second years respectively. Mortality rates did not differ significantly between years. Production varied from 0.099 g (AFDW) m⁻² year⁻¹ to 0.135 g m⁻² year⁻¹ while the ratio between production and biomass (P/B) ranged from 1.75 to 1.55. The larger body size of *N. virginea* may account for its low P/B compared to hydrobiids and *Bittium* spp.

Chapter 2 - Mating, oviposition, post-oviposition period, development, and generation period of the two land snail species *Monacha cartusiana* (Müller) and *Eobania vermiculata* (Müller) were studied in the laboratory in Egypt during the two seasons of 2003/4 and 2004/5. Data revealed that mating is essential for both snail species to lay eggs, thus it occurs when snails reached their sexual maturity (from adulthood till mating) during a period averaged 190.3 and 228.6 days for *M. cartusiana* and 231.6 and 568.3 days for *E. vermiculata* after adulthood in the two different seasons respectively. Pre-oviposition period (from mating till laying first egg) lasted an average of 2.7 and 8.2 days for *M. cartusiana* and 3.5 and 9.2 days for *E. vermiculata* during the two seasons respectively.

Oviposition period (from first laying egg till last one) averaged 22.5 and 64.1 days during which the snail of *M. cartusiana* deposited an average of 63.0 and 177.5 eggs during the two seasons respectively. On the other hand this period averaged 15.2 and 24.9 days for *E. vermiculata* during which the snail individual deposited and total average of 89.6 and 162.0 eggs during 2003/4 and 2004/5 respectively. While, post-oviposition period (from last egg till death) lasted 142.4 and 71.9 days for first snail species and 372.5 and 660.1 days for the

second one during the same seasons respectively. The generation period (from egg to egg) extended an average of 369.8 and 402.6 and 481.7 and 868.7 days for both species during the same seasons respectively.

Chapter 3 - Sundarbans biosphere reserve is a unique ecosystem and characteristic of diverse types of habitats ranging from mudflat to sandy beaches. Nutritionally, economically and ecologically important estuarine edible crab, *Scylla serrata* (Arthropoda: Crustacea: Decapoda) occupy diverse habitats and their survival is dependent on multidimensional immune responses mostly offered by hemocytes – the chief immunoeffector cells of hemolymph. The circulating hemocyte number is a stress indicator and hemocyte counts may be a valuable tool in monitoring the health status in biounsafe environment in *S. serrata*. The enumeration of total and differential hemocyte has been enumerated under the exposure of 1, 2 and 3 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15 and 30 days in controlled laboratory condition. In *S. serrata* diverse subpopulations of hemocytes were identified as hyalinocytes, semigranulocytes and granulocytes. Alteration in hemocyte counts and density of hemocytes were recorded under 1, 2 and 3 ppm of arsenic in static water environment. Shift in THC and DHCs recovered partially in arsenic exposed crabs maintained in arsenic free water up to 30 days. Exposure to 1 ppm of sodium arsenite for 30 days yielded a highest THC as $312.9 \pm 3.6 \times 10^5$ cells / ml against control value of $47.319 \pm 4.8 \times 10^5$ cells / ml. Relatively high THC in the crab maintained in toxin free water for 30 days of span indicates a persistence of cellular stress induced by arsenic exposure. A highest depression in hyalinocyte density was recorded under the exposure of 1 ppm of arsenite for 30 days as 1.17% against control value as 7.47%. Similarly decrease of granulocyte was recorded as 16.21% in 30 days of exposure against control value of 27.24%. Elevation of semigranulocyte was recorded as 82.62% against the control value of 65.29%. Study of possible restoration of DHC was carried out by maintaining the animals in toxin free water for 30 days. All groups of treated animals excepting the groups exposed to 2 ppm for 30 days and 3 ppm for 30 days recovered the DHC of the control. Depression in hyalinocyte and semigranulocyte density was recorded under the exposure of 3 ppm of arsenite for 30 days against control value. Arsenic induced alteration in hemocyte density indicated a state of immunological stress in estuarine crab. Data is suggestive of cellular stress which may lead to decline in endemic population of *S. serrata* in arsenic affected districts of West Bengal.

Chapter 4 - *Lamellidens marginalis* (Mollusca: Eulamellibranchiata) is a freshwater edible mussel distributed in the wetland of different districts of West Bengal of India. Hemocytes – the blood cells of this species perform diverse immunological functions including nonself recognition and phagocytosis of nonself particles. Assay of nonself surface (glass) adhesion of hemocytes was determined under the exposure of 0.006, 0.03, 0.06 and 0.09 ppm of a biopesticide azadirachtin in diverse span of exposure i.e. 1, 2, 3, 4 and 7 days in controlled laboratory condition. Shifts in percentage of adhesion were recorded against all the concentration screened. However, the patterns of shift were not uniform.

Azadirachtin induced shift in surface adhesion of hemocytes indicated a state of immunological toxicity and stress in the species. Phagocytosis of nonself particulates is considered as a classical immunological response in bivalve. We have examined phagocytosis of yeast (*Saccharomyces cerevisiae*) by hemocytes of mussel exposed to 0.006, 0.03, 0.06 and 0.09 ppm of azadirachtin for 1, 2, 3, 4 and 7 days *in vitro*.

Alteration in phagocytic response and adhesion of hemocytes is suggestive of cellular stress which may lead to decline in population of *L. maginalis* in xenobiotic affected districts of West Bengal of India.

Chapter 5 - Sundarbans biosphere reserve of India is an ecologically important zone which shelters diverse forms of animals and plants. *Scylla serrata* (Arthropoda: Crustacea: Decapoda) or estuarine mudcrab is an important consumer of this mangrove ecosystem. Blood or hemolymph of *S. serrata* contains hemocytes, capable of performing diverse physiological functions. Hemocytes the circulating blood cells are considered as immunoreactive agents capable of performing phagocytosis, nonself adhesion and aggregation. Arsenic is a major environmental threat in the specific districts of the state of West Bengal of India. Estuarine water of Sundarbans mangrove bears the risk of arsenic contamination due to anthropogenic and geological consequences. Magnitude of hemocyte aggregation was studied in depth under the exposure of 1, 2 and 3 ppm of sodium arsenite for 1,2,3,4,7,15 and 30 days of exposure. Arsenic exposure yields a dose responsive decrease of hemocyte aggregation against a control level of aggregation of 67.77%. Estuarine ecosystem of Sundarbans mangrove is characteristics of severe intra and interspecific struggle. In this dynamic ecosystem, the inhabitants participate in the struggle of niche occupation for survival and existence. Situation often leads to a state of acute predation and fight among animals. As a result, the animals experience physical wounding and loss of body fluid. Aggregation of hemocyte at wound site prevents the loss of blood and entry of microorganism and considered as an immunological response. Magnitude of aggregation was screened under the experimental exposure of sodium citrate, EDTA, heparin, fructose and mannose at different concentrations. Study was aimed to screen the effect of chelating agent, sugars and anticoagulants on aggregation. For all the chemicals screened, a drastic decrease in the occurrence of free cells were reported which is suggestive to role of these agents in the physiological process of hemocyte aggregation. Moreover exposure to arsenic may lead to gradual loss of blood cell homeostasis of mudcrab distributed in its natural habitat. Continuous exposure to toxic arsenic may lead to a population decline of mud crab and loss of biodiversity in this region.

Chapter 6 - The fat body of female *Periplaneta americana* is the main site of vitellogenins (Vgs) synthesis. The Vgs are large molecules synthesized in the fat body and encoded by two different Vg genes (*Vg1* and *Vg2*). The present study indicates that both Vg genes are represented in the genome by a single copy as revealed by Southern blot analysis.

The *Vg2* gene is predominantly expressed in the fat body cells and its expression preceded that of *Vg1* gene. The expression profile and level of Vg genes are found to be synchronized and up-regulated by the hemolymph JH titer and suppressed by the ecdysteroid titer during vitellogenesis, as revealed by RT-PCR and real time PCR analysis.

The differences in the expression levels of Vg genes, suggest the possible differences between the two genes in their responsiveness to the hemolymph JH titer and will pave the way toward further investigation on the JH responsive elements that regulate the expression of these two genes.

Chapter 7 - The carbohydrate expression of goblet cells in the intestine of pigs, rabbits and horses was characterized using lectin histochemistry. Paraffin embedded intestinal slices were incubated with the following biotinylated lectins: Con-A, WGA, DBA, SBA, PNA, RCA-1 and UEA-1. Except for some rectal goblet cells present in pigs and horses, no reactivity was found to Con-A. On the contrary, reactivity to DBA, WGA and RCA-1 was

heterogeneous in all the intestinal sections of the studied species. PNA reactivity was absent in the small intestine with the exception of few positive cells present in the horse. DBA did not bind to colonic goblet cells in the pig, while SBA did not bind the same cells in pigs and horses. RCA-1 staining was negative in the large intestine of the horse. The lectin binding with UEA-1 differed among different intestinal sections and species, remaining unstained in the small intestine of rabbits, colon of horses and rectum of pigs.

Our results led us to conclude that the lectin binding pattern of the intestinal goblet cells is characteristic of each animal species and shows variations related with the intestinal segment analyzed.

Chapter 8 - The relationship between ciliated fibroblast found in the periodontal ligament of rodent incisor teeth and eruption rate was studied under different eruption conditions, using an estimation of eruption rate and ultra structural aspects of primary cilia. Inferior left incisor teeth were cut for 24 days to increase the rate of eruption. During this period, the rate of eruption doubled; however, after this period, the number and frequency of ciliated fibroblasts was not altered. This lack of change does not necessarily signify that ciliated fibroblast do not have a direct or indirect role in the process of eruption, as suggested by the characteristics of the periodontal ligament structure and emerging functions for the primary cilium found in other tissues.

Chapter 9 - The Gulf of Maranhão is the largest and more important estuarine complex in the state of Maranhão, Brazil. São Marcos Bay located in the northeast region of the gulf, contains many small estuaries. The water circulation is due mainly to the tidal currents. This report describes the structure of the zooplankton community in relation to hydrographic conditions in the area, during the dry season/2000. The zooplankton samples were obtained in horizontal tows using a net with 200 μm mesh size. Temperature, salinity, dissolved oxygen and chlorophyll-*a* were measured in water samples. Temperature and salinity were homogeneous throughout the water column. The highest zooplankton densities were observed in São Marcos Bay (up to 19,500 ind.m⁻³), and the lowest were recorded in the Cachorros River and Coqueiros Strait (less than 8,000 ind.m⁻³). Fifty zooplankton taxa were identified. The copepods, decapods and chaetognaths represented 99% of the total density. *Parvocalanus crassirostris*, *Acartia tonsa*, *Subeucalanus pileatus* and *Oithona hebes* were the most abundant species. *Parasagitta tenuis* and *Ferosagitta hispida* were also abundant. Zooplankton diversity varied from 1.33 to 1.71 bits.ind⁻¹. The diversity index followed the variations in the density, and was similar to those found in other coastal systems of northeast Brazil.

Chapter 10 - Pill millipedes, belonging to the genus *Arthrosphaera* (Family: Sphaerotheriidae) are endemic giant millipedes found mainly in the upper moist soil horizons of the forests of Southern India and Sri Lanka. They are also known as rollers as they roll into sphere or pill on conglobation as defense behavior. The bulldozing shape of body of pill millipedes helps to till and mix the soil with litter fragments, transfer organic matter into soil through burrows and uplift organic matter from underneath to generate moder humus. They hibernate into the subsoil surface on the onset of summer and attain quiescent state. Their large body size confers several advantages like tolerance to elevated moisture stress and increase the burrowing ability. The large body size is also known to affect energy acquisition and utilization, and in turn the rates of organic matter decomposition. The order, Sphaerotheriida characterized by 13 body segments (tergites) and 21 pairs of walking legs. The tergites are strongly curved and horny. Each tergite (except for pygidium) is overlapped

slightly by the preceding tergite. In the process of conglobation, the head and ventral structures are completely covered by the pygidium, which helps in protection of the head and sense organs. Further, special closing ledges (locking carinae) are located on the inner side of the tergites. Stridulation (rubbing the last legs against the sides of last tergite generate sound) is an interesting phenomenon in *Arthrosphaera*. Stridulation behavior was seen usually in females and occasionally in male *Arthrosphaera* of the Western Ghats. The male stridulatory organ is the harp and in female it is washboard located at the anterior telopod and subanal plate respectively. Systematic descriptions of the order Sphaerotheriida are lacking, the current classification scheme lacks sufficient description and demands more morphological features to define monophyletic groups in *Arthrosphaera*. The current study, in addition to available morphological descriptions, emphasizes other essential features to revise the description of four species of *Arthrosphaera* (*Arthrosphaera dalyi*, *A. davisoni*, *A. fumosa* and *A. magna*) found in the Western Ghats along with their distribution pattern. The morphological description such as color, head, antennae, mouthparts, gnathochilarium, collum, thoracic shield, tergites, endotergum, anal shield, legs, female sexual features and male sexual features have been furnished.

Chapter 11 - The molecular phylogeny using mitochondrial DNA sequences provides valuable insights for wildlife conservation. However, the inference of phylogenies may be affected by the marker type as well as by the statistical model applied for data analysis. This study reports a comparative evaluation of neighbor-joining (NJ) and maximum-parsimony (MP) methods for phylogenetic inference among three *Oryx* species (*Oryx leucoryx*, *Oryx dammah* and *Oryx gazella*) as well as two closely related (same subfamily but different genera) outgroups including Addax and Roan, using 12S rRNA and 16S rRNA genes. We observed that the 12S rRNA based phylogenetic inference is unable to differentiate between the genus *Oryx* and Addax, regardless of the method used. However, the 16S rRNA gene segment accurately grouped all the five taxa using MP but not the NJ suggesting the possible superiority of the former method for phylogenetic analysis using similar data structure. This report also describes a stepwise protocol that can easily be followed for constructing the trees using PHYLIP software.

Chapter 12 - An organophosphate pesticide (Methyl parathion) was presented singly and also in combination with two different strains of aeromonads, once at a time, to *Channa punctatus* (Bloch). In case of combined treatments, adult fish were exposed to a sublethal dose (0.001ml/l) of methyl parathion coupled with challenge of sublethal dose (4×10^7 cfu/ml) of either *Aeromonas hydrophila* (MTCC 646) or *Aeromonas salmonicida* (MTCC 1522) strain injected intramuscularly. The bacterial challenge was initiated 5 days after the pesticidal exposure. The experiment comprised measurement of nine haematological parameters at 3, 7, 14, 21 and 28 days of intervals respectively. A steep increase in the total leucocytic population and corresponding leucocrit values were observed initially in combined treatments compared to fish groups exposed only with pesticides. However, all the treated groups generally showed a drop in lymphocyte population (lymphocytopenia) in the blood. Most of the erythrocytic parameters were depressed in fish groups treated only with pesticide while erythrocytic depression was less pronounced in combined treated groups.

Chapter 13 - Two new species each under the genus *Scutascirus* Shiba (Family: Cunaxidae) and *Gamasellodes* Atheis-Henriot (Family: Ascidae) collected from dried mushroom *Pleurotus ostreatus* in West Bengal, India are described and illustrated.

Chapter 14 - The dependency on noninvasive sources of DNA such as feces, hair and feathers for genetic analyses is particularly important in studies where conventional sampling is hard to practice, as in the case of wildlife animals. In view of earlier reports, we hereby address some important issues on merits, demerits and precautions related to the use of noninvasive methods of DNA sampling for molecular markers analysis.

Chapter 15 - Thyroid hormones promote normal growth and development and regulate a number of homeostatic functions in the body (Fujita, 1988). We hypothesized thyroid hormone may have an important role in maintaining the glucose homeostasis and morphology of cardiac muscle and skin in rat. We used methimazole 20 mg/kg and thyroxine 600 µg/kg body weight/day for 14 days intraperitoneally to the experimental groups of rat to induce hypo- and hyperthyroid like conditions respectively. Serum T₃, T₄ hormones were measured by radioimmunoassay (RIA). Serum Glutamic Oxaloacetate Transaminase (SGOT) or Aspartate Amino Transferase (AST) measured by Spectrophotometric method. T₃ and T₄ levels were significantly reduced in hypothyroid rat but increased in hyperthyroid rat as expected. Thyroxine induced hyperthyroid rat exhibited hypertrophy in heart muscle and elevation of serum SGOT level.

Both hypo- and hyperthyroid induction changes the glucose homeostasis as reflected in Insulin Tolerance Test (ITT). The elevated blood glucose level returned to the basal level after 120 minutes of insulin challenge in control rat. But the same does not happened in hypo- and hyperthyroid rat. Consequently methimazole induced thyroid hormone deprivation also changes the skin architecture.

Chapter 16 - The Oncotic cytology appeared from a study developed by George Papanicolaou, with the objective to analyze the cervical squamous cells in all stages of the menstrual cycle periods, becoming a basic mean of prophylaxis for the cervical cancer. The technique consists of the analysis of the slides with cervical smear and of the detection of cellular alterations with pre-malignant or malignant characteristics. During the cytological analysis fungus, parasites, signals of inflammation, among others alterations can be found. The results are formatted following to Bethesda System, to Report of Papanicolau or Brazilian Nomenclature for Report of Cervical Pathologies. This article had the goal to introduce a review about the characteristics of the cervical cancer and the techniques that are used to diagnostic it.

Chapter 17 - Haemocyte mediated non-self phagocytosis and generation of nitric oxide (NO) as reactive nitrogen intermediate were examined to investigate the acceptability of the parameters as biomarkers of sodium arsenite induced stress on *Lamellidens marginalis* (Mollusca; Bivalvia; Eulamellibranchiata), at sublethal concentrations. The animals suffered a remarkable immune compromise by the natural toxicant. Partial recovery was observed in the phagocytic potential and NO generation by the haemocytes of the animals over a period of thirty days. Quantitation of phagocytic efficiency and intrahaemocyte NO generation suggests reliability of the parameters as cellular biomarkers of sodium arsenite induced stress on freshwater molluscs.

Chapter 18 - Spawning of the killifish, *Aphanius dispar dispar*, have been studied in freshwater under controlled laboratory conditions. Two breeding systems, with cages and without cages, were used to study the fish spawning. Five replicates for each system were used. One male and two females were stocked in each tank for ten weeks. The total production of eggs in the tanks with cage system was 144 eggs and the average egg production per tank per week was 2.88 eggs. 59 fries were hatched and the hatchability rate

was 41%. No eggs were found in the tanks without cages probably because of predation by their parents. There was a highly significant ($P < 0.0001$) difference between the two systems in egg production. It was also observed that the eggs were infected with fungus in the breeding and incubation tanks which reduced the eggs production and hatching rate, and caused fry mortality. The present study revealed that the tanks with cage system was the best method to prevent predation of fish on eggs and fry, and to increase fry production. The Ultra Violet (UV) system is recommended to disinfect water of breeding tanks and incubation jars to eliminate fungal infection of eggs. This will help in mass production of *A. dispar dispar* eggs and fry for restocking of freshwater bodies for biological conservation of the fish to be used for biological control of mosquito larvae and ornamental purpose.

Chapter 19 - Polysaccharides from *Phaffia rhodozyma* SDS-treated cell walls were successively obtained via cold water, hot water, and dimethylsulfoxide extractions. For this, processed yeast cells were grown under a physiological condition of active production of astaxanthin, its characteristic carotenoid metabolite. Afterwards, the extracted fractions were fractionated via freeze-thawing and precipitation with either Fehling reagent or Cetavlon. An α -D-glucan and a heteropolysaccharide were isolated and structurally investigated using monosaccharide composition determination, methylation analysis, and COSY, TOCSY and HMQC techniques of ^{13}C - and ^1H -NMR. The DMSO-extracted and Fehling-purified α -D-glucan was determined to be a linear chain of α -D-(1 $\rightarrow$ 3)-glucopyranosyl units. The hot water-extracted and Cetavlon-precipitated heteropolysaccharide showed a complex structure containing α -D-(1 $\rightarrow$ 4)-mannopyranosyl units-based main chain with side chains containing β -D-(1 $\rightarrow$ 4)-xylopyranosyl units along with D-mannose, D-galactose, and D-glucose units. In addition, these monosaccharides are found to be non-reducing ends of the heteropolysaccharide as well. The cell wall extraction schedule also yielded several minor glucan fractions displaying a positive iodophilic reaction which was abolished after pretreatment with crystalline *Bacillus subtilis* α -amylase. The knowledge of these cell wall features is of importance for the application of whole *P. rhodozyma* cells as single cell protein, improved digestibility, and astaxanthin release.

Chapter 20 - Zoonotic visceral leishmaniasis is a re-emerging disease caused by *L. infantum*/*L. chagasi*. The disease is transmitted by phlebotominae sand flies and dogs are the main urban reservoir of the parasite. In the natural history of *L. chagasi* infection in dog, named canine visceral leishmaniasis (CVL), following transmission, the parasites multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog. In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites. After infection, some dogs develop an impaired cell-mediated immune response that permits parasite dissemination and tissue lesion formation (symptomatic dogs), whereas others control parasite proliferation and dissemination to the different tissues (asymptomatic dogs). These infected dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. The cellular basis and mechanisms for the development of T-cell unresponsiveness in CVL are not

understood fully. In the present review it will be discussed the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL.

Chapters 21 and 22 feature abstracts regarding animal science and issues by several different authors.

POPULATION DYNAMICS AND SECONDARY PRODUCTION OF THE SNAIL *NERITINA VIRGINEA* (LINNAEUS, 1758) (MOLLUSCA: GASTROPODA: NERITIDAE) IN AN ESTUARY IN SOUTHERN BRAZIL

Ricardo S. Absalão^{1,3}, Ricardo S. Cardoso^{*1} and Alexandre S. Alencar¹

¹Departamento de Zoologia, Instituto de Biologia,
Universidade do Estado do Rio de Janeiro,
Av. São Francisco Xavier 524, Maracanã,
Rio de Janeiro, RJ. CEP 20550- 011

²Laboratório de Dinâmica de Populações Marinhas,
Depto. Ciências Naturais, Universidade do Rio de Janeiro (UNI-RIO),
Av. Pasteur nº 296 - Urca, Rio de Janeiro-RJ, CEP 22.290-240. Brasil

³Departamento de Zoologia, Instituto de Biologia, C.C.S.,
Universidade Federal do Rio de Janeiro,
Ilha do Fundão, Rio de Janeiro, RJ, CEP. 21941-570 - BRASIL

ABSTRACT

The population of *Neritina virginea* (Linnaeus, 1758) in Barra Grande estuary, Ilha Grande, state of Rio de Janeiro in southern Brazil (23° S) was observed from February 1999 through December 2000. The sandflat where the neritids live was divided into 5 sectors, each 30 m long, and each sector was sampled separately. Densities were higher in spring and lower in winter. Reproduction was continuous year-round, but with increased activity in early spring. Fecundity was about 80 eggs/embryos per capsule, and the sex-ratio was 1 males: 2.89 females. This population of *N. virginea* attained higher densities and shell size in the inner part of the estuary. A total of 40,596 individuals was sampled, with shell length varying between 2.50 and 18.80 mm. Life span varied between 1.78 and 2 years for the first and second years respectively. Mortality rates did not differ significantly between years. Production varied from 0.099 g (AFDW) m⁻² year⁻¹ to 0.135 g m⁻² year⁻¹ while the ratio between production and biomass (P/B) ranged from 1.75 to 1.55. The larger body size of *N. virginea* may account for its low P/B compared to hydrobiids and *Bittium* spp.

* E-mail: rcardoso@unirio.br. Tel. +55-21-22295-3436, Fax +55-21-22295-3436.

Keywords: Population biology - Life history - Growth - Mortality - Productivity – Neritidae, *Neritina virginea*, Neritidae, population biology, life history, mortality, productivity.

INTRODUCTION

The family Neritidae contains about 50 species. These snails are selective grazers (LEHMAN; HAMILTON, 1980), and are generally associated with marine grasses (*Spartina*, *Ruppia*, etc.), playing an important ecological role affecting decomposition and nitrogen recycling in the salt marsh (KEMP et al., 1990). There are numerous studies on their anatomy and physiology (e.g. BOURNE 1908, 1911; ANDREWS, 1937; FRETTER, 1965, 1966, 1984, HIROKI, 1978), aspects of reproduction (ANDREWS, 1935, 1937; HOUSTON, 1990; MARTINS, 1992), and systematics (BAKER, 1923; FLORES; CÁRCERES 1973; MATTHEWS-CASCON et al., 1992).

Few works were been done on the genus *Neritina*. Murty; Rao (1978) showed that *Neritina violacea* had conchological differences among sub-populations living in intertidal habitats. Huang (1997) found morphological, allozyme, and karyotypic differences between *N. violacea* and *N. cornucopia*. Lane (1991) and Underwood (1984), working on *N. reclivata* and *Nerita* and *Bembicum* respectively, showed that they have a strong grazing effect on the epiphytic film on marine grasses. Adegoke et al. (1969) provided a superficial approach to the population dynamics of sympatric neritid populations on the Nigerian coast, reporting sediment preferences and spawning time; and Alpírez et al. (1984) reported on some predators of *Neritina latissima*.

Neritina virginea (Linnaeus 1758) is a common and abundant snail in estuaries, salt marshes, and intertidal mud and sand flats. It is found widely in the Americas from North Carolina, USA (36°N) to Santa Catarina, Brazil (29°S; RIOS, 1994), and also occurs in Bermuda and the Antilles (WARMKE; ABBOTT, 1962). Despite its broad geographical and ecological range, we know little about this species. However, a few studies have shown that *N. virginea* is a common component of certain benthic communities (TARARAM; WAKABARA, 1987; FLYNN, 1993; LANA; GUISS 1991, 1992; LANA et al., 1991). The present work is the first to estimate the population parameters (growth, mortality, recruitment), reproductive period, production, and turnover of *Neritina virginea*.

MATERIAL AND METHODS

Study Area

The study was carried out in Dois Rios bay (23°11'24"S, 44°11'36"W) Biological Reserve, Ilha Grande, state of Rio de Janeiro. Ilha Grande is the largest island off the coast of Brazil, 13 km wide and 36 km long. The climate is hot and humid (annual mean temperature around 22.5°C, annual precipitation 2500 mm). The Dois Rios Bight has two rivers reaching the sea, one on each side of the inlet. The larger is Barra Grande River, which is about 1 km long in the sedimentary plain. The amount of fresh water varies according to the intensity of the rainy season, and salt water extends about 0.5 km into the estuary.

In the estuary is a sandy intertidal flat that extends over 150 m and is located 250 m from the river mouth. Fine marine sand occurs in the first 2 sectors (nearest the river mouth), sector 3 has a mix of fine marine sand and medium to coarse river sand, sector 4 is composed of coarse river sand with a bit of biotrital calcium carbonate, and sector 5 has coarser river sand with much biotrital calcium carbonate (ABSALÃO; CARDOSO in prep).

Sampling Procedure

The population of *Neritina virginea* in the Barra Grande River estuary was sampled monthly from February 1999 through December 2000, always at low tide. The sandflat inhabited by the gastropods was divided into 5 sectors each 30 m length, and each sector was sampled separately. Each sandflat sector was scrutinized for 20 min by 3 collectors. During that time period, all individuals seen were caught by hand, placed in a plastic bag with local water, and immediately transported to the laboratory where the lengths of the snails were measured with calipers.

All specimens were then returned to their respective sectors, except the ones utilized to calculate the secondary production ($n=160$). The characteristics of the surface sediment in each sector were determined. Five (replicates) 10-cm-deep cores (5.5 cm Ø) were collected in each sector, in each season. Because the silt-clay fraction was insignificant, all sediment was sieved. Salinity in each sector, in each season of the year, was measured with a salinometer.

Growth

To perform the growth analysis, the ELEFAN (Electronic Frequency Analysis) routine of the FISAT package (FAO-ICLARM Stock Assessment Tools) was used. This approach assumes that growth is described by the von Bertalanffy model modified for seasonality (GAYANILO et al., 1996): $L_t = L_\infty [1 - e^{[-K(t-t_0) + (KC/2\pi) \sin 2\pi(t-WP) - (KC/2\pi) \sin 2\pi(t_0-WP)]}]$, where L_t is length at time t (mm); L_∞ is the theoretical maximum length attained by the species; K is the curvature parameter indicating the growing velocity of the population, according to von Bertalanffy model; C is a constant for the amplitude of oscillation in seasonal growth; t_0 is age at zero length; and WP (winter-point, i.e. the period of growth reduction, expressed as a decimal fraction of the year). The graphical representation of this equation produces a curve of which the agreement is represented by the R_n value (goodness of fit index), which is analogous to the parameter r^2 in correlation analysis and varies from 0 to 1 (GAYANILO et al., 1996).

The growth index phi prime (ϕ') defined as: $\phi' = 2 \log_{10} (L_\infty) + \log_{10} K$ (PAULY; MUNRO, 1984) was used to measure growth performance. This criterion was chosen because the negative correlation between K and L_∞ (PAULY; MUNRO, 1984) invalidates comparisons based on individual parameters.

Mortality

Total mortality (Z) was calculated from the length-converted catch curve (PAULY et al., 1995) of the ELEFAN II routine of the FISAT program (GAYANILO et al., 1996). The estimation of Z was given by: $\ln(N) = a - Z * t$, where N is the number of individuals; a the regression intercept, Z , with sign changed, the unbiased mortality estimated and t is the estimated age in each cohort. (PAULY et al., 1995). An analysis of covariance (ANCOVA) was employed to compare the mortality between years, using age as the covariate. Life span was estimated considering $L_{99\%}$ as the length at which 99% of the population was represented (ALAGARAJA, 1984). The annual finite mortality rate (A) was obtained from the expression: $A = 1 - e^{-Z}$ according to Ricker (1975).

Secondary Production

The relationship between length and ash free dry weight was estimated by a power function of the form $W = a * L^b$, where W is the monthly mean ash-free dry weight per individual (mg); L is the length of the size class (mm); and a and b are constants. Production was estimated by the weight specific growth rate method. In these calculations it is necessary to measure growth or mortality rates as a function of size and season and to carry out a summation throughout the year for each size class (CRISP, 1984). The production is given by the equation: $P = \sum \sum f_i * G_i * w_i * \Delta t$, where f_i is the mean number of individuals of this size class existing in the population during the period Δt , G_i is the weight specific growth rate of size class i , w_i the mean weight of the size class and Δt is the interval of time. G_i can be obtained by $G_i = b K [(L_{\infty} - L_i)/L_i]$, where b is the exponent of the length-weight relationship; K and L_{∞} are VBGF parameters and L_i is the mean length in length class i .

Annual mean biomass was calculated as follows : $B = \sum \sum f_i * w_i * \Delta t$

Reproductive Activities

Reproductive activities were estimated indirectly, because the snail population is located in a biological reserve and it was not possible to kill a large number of animals. We observed copulating pairs of snails throughout the year, but did not count them; rather we recorded the number of snails bearing egg capsules on their shells. Utilizing a similar indirect procedure, we measured the shell length of several pairs of copulating animals; the length of the smallest ones was considered as the minimum size to sexual maturity. The sex ratio was established by dissecting 214 randomly collected specimens.

Statistical Analysis

The χ^2 test was used to test the proportion of males to females. One-way ANOVA and Tukey's multiple range test were performed to analyze the distribution of abundance and length classes and also the water salinity in each sampling sector (ZAR, 1996). Data of

population abundance were tested for normality and homoscedasticity and compared ANOVA. A root-root transformation was employed to stabilize variances.

RESULTS

Population Structure

The smallest individual collected was 2.4 mm, whereas the largest individual measured 18.5 mm. The population of *N. virginea* reached its highest densities in the spring (October 1999 and September 2000). The lowest densities were recorded in winter (July 1999 and 2000) (Figure 1). The apparent lower values from the first three observations in 1999 reflect the influence of oceanographic and hydrographic conditions on the collecting. During that period, sand had accumulated on the Barra Grande bar, narrowing the connection between the river and the sea, so that the water level in the estuary was unusually high, even at low tide when we were sampling.

During all sampling periods, specimens were found bearing egg capsules (Figure 1), suggesting that there is continuous reproductive activity year-round but increasing in spring (October 1999 and 2000). The regression equation for the number (x) of egg capsules on the shell versus the total population density (y) is expressed by: $y = 275.84 + 0.21x$; $n = 19$, correlation coefficient $r = 0.69$ ($p < 0.01$). The fecundity of *N. virginea* was about 80 eggs or embryos per capsule (mean = 81, sd = 21, $n = 62$). The smallest mating specimens sized 6.9 mm for males and 8.6 mm for females.

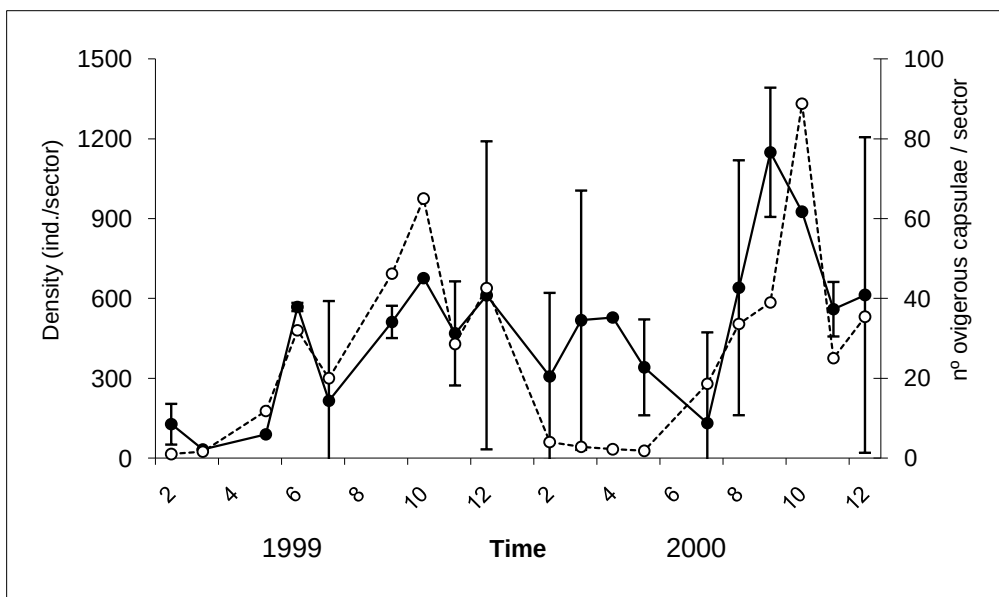


Figure 1. *Neritina virginea*. Population density (full circles) and number of ovigerous capsules (open circles) for period February 1999 to December 2000. Vertical bars represent standard deviation of population density.

The male:female ratio was 1:2.89 ($\chi^2 = 50.54$; $n = 214$, $p < 0.01$). There was a weak but significant relationship between shell length and the number of egg capsules ($r = 0.16$, $n = 2403$, $p < 0.01$). The high standard deviation in Figure 1 suggests a clumped distribution of individuals, mainly in sectors 4 and 5, the innermost part of the estuary (Table 1b).

Table 1(a). Analysis of variance. One-way ANOVA. Relation between sampling sector and abundances of *Neritina virginea*. df = degrees of freedom. p = probability level. (b) Results of Tukey’s multiple range test analysis undertaken as an a posteriori test of multiple comparisons to indicate statistical significance

(a)	df	sum of squares	mean squares	F	p
Between sector	4	4763835.3	1190958.8	11.57	0.00
Within sector	82	8437927.2	102901.6		
Total	86	13201762			
(b)					
Sampling sector	1	2	3	4	5
Mean density	185.39	229.18	472.82	677.47	767.11
Non-significance					

Highest abundances were observed in sectors 4 and 5 (Figure 2). This was confirmed by one-way ANOVA and Tukey’s multiple range test (Table 1a and b). There was stratification by size. There was a significant correlation between mean length and spatial location of the individuals (Table 2a). Smaller individuals were more numerous in sectors 1 and 2, and larger ones in sectors 4 and 5 (Table 2b, Figure 2).

There were no significant differences in salinity among sectors of the sandflat ($F = 2.44$, $p > 0.05$, $df = 4/125$).

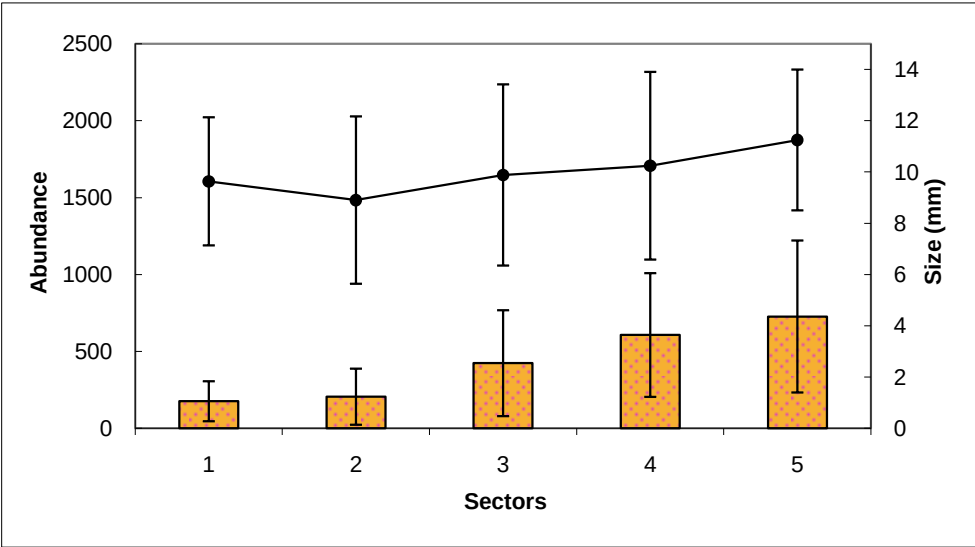


Figure 2. *Neritina virginea*. Spatial variability of population abundance (bar) and shell mean size (mm) (line) along sampling sectors. Vertical bars represent standard deviation.

Table 2(a). Analysis of variance. One-way ANOVA. Relation between sampling sector and shell mean length of *Neritina virginea*. df = degrees of freedom. p = probability level. (b) Results of Tukey's multiple range test analysis undertaken as an a posteriori test of multiple comparisons to indicate statistical significance

(a)	df	sum of squares	mean squares	F	p
Between sector	4	47.46	11.86	21.74	0.00
Within sector	82	44.75	0.54		
Total	86	92.20			
(b)					
Sampling sector	1	2	3	4	5
Mean length	10.17	9.95	11.04	11.45	11.87
Non-significance					

Growth

A total of 40,596 individuals was sampled, with lengths varying between 2.50 and 18.80 mm. The $L_{99\%}$ (15.84mm) did not differ between the two sampling years, while L_{∞} varied less between years (Table 3). Year 1 had a higher curvature parameter (K) than year 2 (Table 3). The values for parameter C (degree of seasonal variation) varied between years. In year 1, C was equal to 1, indicating a halt in growth, while in year 2, C equaled 0.65, suggesting a slight oscillation in growth. WP (the point of lowest growth rate during the year) differed between years; WP in year 1 showed a decrease in growth rate at the end of July, but in September in year 2.

The life span of *N. virginea* in year 1 (1.78 years) was shorter than in year 2 (2 years), being a function of the higher curvature parameter of growth and of L_{∞} in year 1. Longevity, based on the maximum measured length (18.00 mm), was between 3.55 and 3.85 years for years 1 and 2 respectively. Growth intensity was higher in the first year, as indicated by the estimator ϕ' (Table 3).

Table 3. *Neritina virginea*. Von Bertalanffy growth function (VBGF) parameters: L_{∞} (mm) = asymptotic length; K (year⁻¹) = curvature parameter, C = constant for the amplitude of seasonal oscillation of growth; WP = point of lowest growth rate in the year; Rn (goodness of fit) = agreement of the estimated observed growth rates. $L_{99\%}$ = length (mm) at which 99% of the population was represented and life span estimated for years. ϕ' = Growth index phi prime

Period	L_{∞} (mm)	K (year ⁻¹)	C	WP	$L_{99\%}$	Life span (mo)	Rn	ϕ'
1 year	18.6	1.15	1.0	0.65	15.84	21.36	0.364	2.60
2 year	18.5	0.93	0.6	0.79	15.84	24.00	0.225	2.50

Mortality

In year 1, instantaneous mortality was lower ($Z = 3.47 \text{ year}^{-1}$) than in year 2 ($Z = 3.99 \text{ year}^{-1}$). However, instantaneous mortality rates showed no significant differences when both sampling years were compared (Table 4). The annual finite mortality rate (A) of *N. virginea* was 0.97 year^{-1} and 0.98 year^{-1} respectively for years 1 and 2.

Table 4. *Neritina virginea*. Mortality estimates (Z) for 1st and 2nd year, and associated statistics (ANCOVA)

Parameters	1 st year	2 nd year
	Mean (SD)	Mean (SD)
a	10.49 (0.86)	11.88 (0.55)
Z	3.47 (0.32)	3.99 (0.23)
R ²	0.92	0.98
N	13	9
ANCOVA $F_{1,18} = 0.78$; $p > 0.05$		

Secondary Productivity

The regression equation between mean weight and length classes for the population of *Neritina virginea* was: $W_{(ash-free \text{ dry weight})} = 6 * 10^{-5} * L_{(mm)}^{2.4415}$ (n=128 and $r=0.99$, $p<0.001$).

Mean biomass was $0.057 \text{ g AFDW m}^{-2}$ for year 1, and $0.087 \text{ g AFDW m}^{-2}$ for year 2. The estimated production was $0.099 \text{ g AFDW m}^{-2} \text{ year}^{-1}$ and $0.135 \text{ g AFDW m}^{-2} \text{ year}^{-1}$ respectively for years 1 and 2. The ratio between production and biomass (P/B) was 1.75 and 1.55 for years 1 and 2 respectively.

DISCUSSION

Population Fluctuation

Fluctuations in population densities are frequent in mollusk species of many shallow marine habitats (CAPEHART; HACKNEY, 1989; ARIAS; DRAKE, 1994; HECK et al., 1995; SARDÁ et al., 1995; MITRI; ROSSI, 1999). The population of *N. virginea* in the Dois Rios Bight (23°11'S) reached higher densities in spring, partially agreeing with observations by Hernández; Lanza (1989), who found higher densities of *N. virginea* during spring and summer in a lagoon system in Mexico (18°N). On the other hand, Lana; Guiss (1991), in a salt marsh in southeastern Brazil (25°S), found higher densities of *N. virginea* during winter. Bonnet et al. (1994), working in the same salt marsh as Lana; Guiss (1991), found no seasonal differences in the density of *N. virginea*. The different climates of the sites studied may explain part of the observed variability. Although the data of Bonnet et al. (1994) might indicate an annually variable life cycle, they are rather the result of an inappropriate sampling program, with only one observation in summer and another in winter.

Virnstein (1980) stated that plant structures provide complexity in the habitat, protection from predators, and food availability. Although *Ruppia maritima* occur in Barra Grande estuary, in quantities up to 74g m⁻² (CREED pers. com.) of production, the distribution of the *N. virginea* population is not affected by the presence of this marine grass, and the snails occur indiscriminately inside and outside the grass patches. By other side, *Spartina* is known by affects hydrodynamic and sedimentary conditions, favoring or impairing the invertebrate fauna (NETTO; LANA, 1997). Lana; Guiss (1991) and Aron (1989) reached similar conclusions about population regulation of *N. virginea*, which physically depends on the habitat provided by stalks and leaves of *Spartina alterniflora*, being used more as a physical substratum than as food. Adegoke et al. (1969) showed that the density of *Neritina glabrata* decreased appreciably during low-salinity months; however, we were unable to find any relationship between precipitation or salinity and the density of *N. virginea*.

Reproduction

Fluctuations in population density of *N. virginea* are related to spawning periods. The correlations obtained herein between the number of egg capsules and the mean density ($r = 0.69$) showed that up to 47.5 % of the population fluctuation can be explained by variations in the number of egg capsules. *N. virginea* lays its egg capsules on a broad spectrum of substrata such as small rocks, weeds, empty and occupied mollusk shells, shell fragments, and leaves of *Ruppia maritima*; but the number of egg capsules on occupied shells is a good indicator of overall reproductive activity. Figure 1 shows continuous production of egg capsules during the entire year, with lowest production in late summer and early autumn and highest in October for both years. On other hand, Andrews (1935) found *N. virginea* in the northern hemisphere breeding in July (summer). Apparently other gastropod inhabitants of estuaries and coastal lagoons show patterns similar to that of the Barra Grande *N. virginea* population (DRAKE; ARIAS, 1995; LILLEBO et al., 1999), with continuous reproduction but with a strong increase in one or two annual periods, probably in the most favorable environmental period. In Barra Grande, the general abundance of egg capsules was strongly correlated with the abundance of shells bearing capsules. Andrews (1935) found that up to 30 % of the shells of *N. virginea* and *Neritilia succinea* and nearly 100% of *Neritina punctulata* bore egg capsules. In the Barra Grande *N. virginea* population, about 5% of all specimens carried egg capsules. The lower percentage of specimens with egg capsules than that reported by Andrews (1935) probably reflects the greater availability of appropriate substrata in Barra Grande.

The fecundity of the Barra Grande population of *N. virginea* (ca. 80 embryos) is within the range (30 to 215) shown by this species in several other environments (ANDREWS, 1935).

Distribution

Neritina virginea reached higher densities and shell size in the inner sectors of the estuary. The greater abundance in sectors 4 and 5 suggests a relationship with the granulometry of the sediment, which becomes coarser farther up the estuary. These results do not accord with those of Aron (1989), Lana; Guiss (1991), and Bonnet et al. (1994), who found populations of *N. virginea* living on muddy substrata. However, *N. virginea* is able to

live on a broad spectrum of sediments, from muds to hard substrata (ANDREWS, 1940). In the above cases, the *Neritina* populations were living associated with *Spartina*, under which they find shelter against desiccation and predators, and on which they find food. However, in Barra Grande there is no *Spartina*, and the occurrence of *Ruppia* is sporadic and variable; therefore the neritids seek their food directly on the substratum and the coarser sand would be more appropriate for the scraping activity of their radulae.

Salinity did not vary significantly among the sectors of the Barra Grande estuary sandflat ($F = 2.44$, $p > 0.05$, $df = 4/125$) where *N. virginea* was found, and therefore cannot be invoked to account for the distribution patterns.

Growth and Life Span

The values of K for *N. virginea* varied between 0.93 and 1.15 year⁻¹. These are intermediate among K values found for other species such as *Hydrobia ulvae* (0.73 year⁻¹; LILLEBO et al., 1999) and *H. ventrosa* (2.6-3) and *H. minoricensis* (1.9-2.1; DRAKE; ARIAS 1995). Probably these differences are related to the different latitudes of the areas studied, the tolerances of each species, coexistence between equivalent species in the same habitat, and the methods used to calculate growth. Growth intensity is best compared using ϕ' , this index shows higher values for *N. virginea* (ca. 2.5) than in hydrobiid snails of temperate regions (*H. ulvae*, $\phi' \cong 1.6$ (LILLEBO et al., 1999), *H. ventrosa*, $\phi' \cong 1.0$ and *H. minoricensis*, $\phi' \cong 1.5$ (DRAKE; ARIAS, 1995). These differences may be explained by latitudinal gradients, since there is a marked tendency toward an increase in growth in tropical regions. The growth rate of *N. virginea* decreases in winter, the same pattern observed in *H. ulvae* by Lillebo et al. (1999).

The decrease in growth rate recorded at the end of July 1999 and in September 2000 (WP 0.65 and 0.79 respectively) suggests a shift in energy spent in somatic growth towards maximum reproduction in October (see Figure 1) as noted by the number of specimens with egg capsules. This was the same growth-reproduction pattern shown by Omena; Amaral (2000) for the polychaete *Laeonereis acuta* in southern Brazil.

Neritina virginea showed a life expectancy between 21 and 24 months. Lillebo et al. (1999) found similar results for *H. ulvae* in an estuary in Portugal (21 months), while Drake; Arias (1995) found a hardly similar period for *H. minoricensis* (18 months).

Mortality

The higher instant mortality tax ($Z = 3.47$ and 3.99 for years 1 and 2 respectively) estimated for *N. virginea* compared to that of *Bullia rhodostoma* ($Z = 2.41$) (McLACHLAN; VAN DER HORST, 1979) can be understood as a higher predatory pressure on the former species. Supposedly, sandy beach ecosystems are strongly controlled by physical factors, relegating biological interactions to second place (McLACHLAN 1996; McLACHLAN et al., 1993). Although the estuarine environment is subject to physical forces such as the rise and fall of the tides and the blending of salt and fresh water, more potential predators of *N. virginea* such as crabs of the genus *Callinectes* are common in this estuary, reaching 12 cm

carapace width, while in sandy beaches (where *Bullia rhodostoma* lives) the hydrodynamics preclude or impede predators. It is usual to find *N. virginea* specimens with signs of predatory activity among the Barra Grande population. Moreover, *N. virginea* is mainly an epifaunal species, while *B. rhodostoma* is infaunal. This may explain the greater exposure of *N. virginea* to predators than *B. rhodostoma*. Also, *N. virginea* is a grazer and would be subject to higher mortality rates than *B. rhodostoma*, a carnivorous species.

Secondary Productivity and Turnover

The values for secondary production obtained for *N. virginea* (between 0.099 and 0.135 g AFDW m⁻² y⁻¹) were of the same order of magnitude as estimates for *Bittium varium* (0.2 g DW m⁻² y⁻¹) by Fredette et al. (1990) and *Hydrobia ulvae* (0.8 g AFDW m⁻² y⁻¹) by Drake; Arias (1995). But they are much lower than estimates for other hydrobiids; for instance, *H. ulvae* varied from 15.2 to 93.7 g AFDW m⁻² y⁻¹ along an eutrophication gradient (LILLEBO et al., 1999). Also, Sola (1996) showed that the same population of *H. ulvae* can show wide variations in secondary production from year to year.

The optimum parameter to compare productivity among species, populations, and communities would be the turnover rate (P/B) (ANSELL et al., 1978). This rate represents the mean value for growth in biomass for a single individual from a given population (BENKE, 1996). The estimates for *N. virginea* P/B were lower than estimates for other estuarine species such as *H. ulvae*, between 1.98 and 5.8 (DRAKE; ARIAS 1995; SOLA, 1996; LILLEBO et al., 1999), *H. ventrosa* of 2.1, and *H. minoricensis* of 2.1 (DRAKE; ARIAS, 1995), *B. varium* 3.2 (FREDETTE et al., 1990), and *B. reticulatum*, between 2.09 and 3.16 (YERRO, 1984). According to Brey; Clarke (1993) and Tumbiolo; Downing (1994), populations with high P/B would be composed of small individuals, with accelerated growth and low life expectancy; while a low P/B would be associated with larger individuals, with low growth and high longevity. The low P/B of *N. virginea* might be related to its large body size compared to hydrobiids and *Bittium* spp.

ACKNOWLEDGMENTS

Thanks are given to all participants in the field work, especially to the biology student Renan Louro. We thank Dr J.W. Reid for English language revision. To CEADS (Centro de Estudos Ambientais e Desenvolvimento Sustentado) for field support and to CNPq for partial financial support to RSA. Finally we would like to thank to Dr^a V. G. Veloso for her suggestions to ms.

REFERENCES

Adegoke, OS; Dessauvagie TFI; Yoloye, VLA. (1969). Biology and population dynamics of two sympatric species of *Neritina* from southern Nigeria. *Malacologia*, 9, 47-51.

- Alagaraja, K. (1984). Simple methods for estimation of parameters for assessing exploited fish stocks. *Indian Journal of Fisheries*, 31, 177-208.
- Alpírez, O; Ureña, JM; Brenes, R. (1984). Hallazgo de un depredador de *Neritina latissima* (Gastropoda : Neritidae) en el río Punta Mala, Puntarenas, *Costa Rica*. *Revista de Biología Tropical*, 32, 159–160.
- Andrews, E.A. (1935). The egg capsules of certain Neritidae. *Journal of Morphology*, 57, 31-59.
- Andrews, E.A. (1937). Certain reproductive organs in the Neritidae. *Journal of Morphology*, 61, 525-559.
- Andrews, E. A. (1940). The snail, *Neritina virginea*, L., in a changing salt pond. *Ecology*, 21, 335-346.
- Ansell, AD.; McLusky, DS; Stirling, A; Trevallion, A. (1978). Production and energy flow in the macrobenthos of two sandy beaches in south west India. *Proceedings of the Royal Society of Edinburgh*, 76B, 269-296.
- Arias, AM; Drake, P. (1994). Structure and production of the benthic macroinvertebrate community in a shallow lagoon in the Bay of Cádiz. *Marine Ecology*, 115, 151-167.
- Aron, AM. (1989). Aspectos ecológicos e comportamentais de *Neritina virginea* (Gastropoda: Prosobranchia) na região estuarino-lagunar de Iguapé – Cananéia – SP. M.Sc. Thesis, Instituto Oceanográfico da Universidade de São Paulo.
- Baker, HB. (1923). Notes on the radula of the Neritidae. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 75, 117-178.
- Benke, AC. (1996). Secondary production of macroinvertebrates. In: *Methods in Stream Ecology*. (F.R. Hauer; G.A. Lamberti, eds), 557-578. London, Academic Press.
- Bourne, GC. (1908). Contributions to the morphology of the group Neritacea of Aspidobranch Gastropods. - Part I. The Neritidae. *Proceedings of Zoological Society*, 2, 810-887.
- Bourne, GC. (1911). Contributions to the morphology of the group Neritacea of Aspidobranch Gastropods. - Part II. The Helicinidae. *Proceedings of Zoological Society*, 5, 759-809.
- Bonnet, BRP; Lana, PC; Guiss, C. (1994). Influência da gramínea *Spartina alterniflora* sobre a distribuição e densidade de *Neritina virginea* (Gastropoda : Neritidae) em marismas da Baía de Paranaguá (Paraná, Brasil). *Nerítica*, 8, 99-108.
- Brey, T; Clarke, A. (1993). Population dynamics of marine benthic invertebrates in Antarctic and subantarctic environment: Are there unique adaptations? *Antarctic Science*, 5, 253-266.
- Capehart, AA; Hackney, CT. (1989). The potential role of roots and rhizomes in structuring salt-marsh benthic communities. *Estuaries*, 12, 119-122.
- Crisp, DJ. (1984). Energy flow measurements. In: *Methods for the study of marine benthos*. (N.A. Holme and A. D. McIntyre, eds). 284-372. Oxford, Blackwell Publ.
- Day, JW Jr.; Smith, WG; Wagner, PR; Stowe, WC. (1973). Community structure and carbon budget of a salt marsh and shallow bay estuarine system in Louisiana. Center for Wetland Research, Louisiana State University. Publ. No. LSU – SG – 72 – 04, 1–80.
- Drake, P; Arias, A M. (1995). Distribution and production of three Hydrobia species (Gastropoda : Hydrobiidae) in a shallow coastal Lagoon in the Bay of Cadiz, Spain. *Journal of Molluscan Studies*, 61, 185–196.

- Flores, C; Cárceles, R. (1973). La familia Neritidae (Mollusca : Archaeogastropoda) en la aguas costeras de Venezuela. *Boletín del Instituto Oceanográfico de la Universidad del Orient*, 12, 3-13.
- Flynn, MN. (1993). Aspectos ecológicos das associações de espécies e avaliação do efeito da predação sobre a estrutura da macrofauna bentônica de bancos de *Spartina* (Cananélia, SP. Brasil). PhD Thesis, Instituto Oceanográfico da Universidade de São Paulo.
- Fredette, T J; Diaz, R J; Van Montfrans, J; Orth, R J. (1990). Secondary production within a seagrass bed (*Zostera marina* and *Ruppia maritima*) in lower Chesapeake Bay. *Estuaries*, 13, 431-440.
- Fretter, V. (1965). Functional studies of the anatomy of some neritid prosobranchs. *Journal of Zoology*, 147, 46-74.
- Fretter, V. (1966). Some observations on neritids. *Malacologia* ,5, 79-80.
- Fretter, V. (1984). The functional anatomy of neritacean limpet *Phenacolepas omanensis* and some comparison with *Septaria*. *Journal of Molluscan Studies*, 50, 8-18.
- Gayanillo, FC.Jr; Sparre, P; Pauly, D. (1996). The FAO-ICLARM stock assessment tools (FISAT) user's guide. *FAO Computerized Information Series (Fisheries)* 8, 126 pp.
- Heck, LK; Able, KW; Roman, CT; Fahay, MP. (1995). Composition, abundance, Biomass, and production of macrofauna in a New England estuary : Comparisons among eelgrass meadows and other nursery habitats. *Estuaries*, 18, 370-389.
- Hernandez, C T; Lanza Espino, G. (1989). Contribución a la biología de *Neritina virginea* (Mollusca) en comunidades de pasto marino *Ruppia maritima* L., (Ruppiacea) en el sistema lagunar de Alvarado, Veracruz, México. *Annales del Instituto de Biología de la Universidad Nacional Autónoma del México, Ser. Zoología*, 60, 143-158.
- Hiroki, K. (1978). Resistance of marine gastropods to oxygen deficiency and hydrogen sulphide. *Boletim de Fisiologia Animal*, 2, 33-42.
- Houston, RS. (1990). Reproductive systems of neritimorph archaeogastropods from the eastern Pacific with special reference to *Nerita funiculata* Menke, 1851. *Veliger*, 33, 103-110.
- Huang, Q. (1997). Morphological, allozymic, and karyotypic distinctions between *Neritina* (*Dostia*) *violacea* and *N. (D.) cornucopia* (Gastropoda : Neritoidea). *Journal of Zoology*, 241, 343-369.
- Kemp, PF; Newell, SY; Hopkinson, CS. (1990). Importance of grazing on the salt-marsh grass *Spartina alterniflora* to nitrogen turnover in a macrofaunal consumer, *Littorina irrorata*, and to decomposition of standing-dead *Spartina*. *Marine Biology*, 104, 311-319.
- Kneib, RT. (1984). Patterns of invertebrate distribution and abundance in the intertidal salt marsh : causes and questions. *Estuaries*, 7, 392-412.
- Lana, PC; Guiss, C. (1991). Influence of *Spartina alterniflora* on structure and temporal variability of macrobenthic associations in a tidal flat of Paranaguá Bay (southeastern Brazil). *Marine Ecology*, 73, 231-244.
- Lana, PC; Guiss, C; Disaró, ST. (1991). Seasonal variation of biomass and production dynamics for above and belowground components of a *Spartina alterniflora* marsh in the euhaline sector of Paranaguá Bay (SE Brazil). *Estuarine Coastal and Shelf Science*, 32, 231-241.
- Lana, PC; Guiss, C. (1992). Macrofauna -plant-biomass interactions in a euhaline salt marsh in Paranaguá Bay (SE Brazil). *Marine Ecology*, 80, 57-64.

- Lane, JM. (1991). The effect of variation in quality and quantity of periphyton on feeding rate and absorption efficiencies of the Snail *Neritina reclivata* (Say). *Journal Experimental Marine Biology and Ecology*, 150, 117-129.
- Lehman, H K; Hamilton, P V. (1980). Some factors influencing the distribution of *Neritina reclivata*. *Northeast Gulf Science*, 1, 67-72.
- Lillebo, AI; Pardal, M A; Marques, JC. (1999). Population structure, dynamics and production of *Hydrobia ulvae* (Pennant) (Mollusca: Prosobranchia) along a eutrophication gradient in the Mondego estuary (Portugal). *Acta Oecologica*, 20, 289-304.
- Martins, IX. (1992). Observações sobre a reprodução, morfologia e sistemática de *Neritina virginea* (Linnaeus, 1758) (Mollusca : Gastropoda : Neritidae). BS. thesis, Universidade Federal do Ceará.
- Matthews-Cascon, H; Pinheiro, PRC; Matthews, HR. (1992). A família Neritidae no Norte e Nordeste do Brasil (Mollusca : Gastropoda). *Caatinga*, 7, 44-56.
- McLachlan, A. (1996). Physical factors in benthic ecology: effects of changing sand particle size on beach fauna. *Marine Ecology*, 131, 205-217.
- McLachlan, A; Van der Horst, G. (1979). Growth and reproduction of two mollusks from an exposed sandy beach. *South African Journal of Zoology*, 14, 194-201.
- McLachlan, A; Jaramillo, E; Donn, T; Wessels, F. (1993). Sandy beach macrofauna communities and their control by the physical environment: a geographical comparison. *Journal of Coastal Research*, 15, 27-38.
- Mistri, M; Rossi, R. (1999). Structure and production of the benthic macroinvertebrate community in a shallow lagoon of the Po River delta. *Italian Journal of Zoology*, 66, 361-368.
- Murty, A S; Rao, M B. (1978). Effect of environment on the shell shape of a tropical estuarine snail *Neritina violacea* (Gmelin) (Gastropoda : Neritacea). *Journal of Molluscan Studies*, 44, 265-271.
- Netto, SA; Lana, PC. (1997). Influence of *Spartina alterniflora* on superficial sediment characteristics of tidal flats in Paranaguá Bay (South-eastern Brazil). *Estuarine Coastal and Shelf Science*, 44, 641-648.
- Omena, EP; Amaral, A C Z. (2000). Population dynamics and secondary production of *Laeonereis acuta* (Treadwell, 1923) (Nereididae : Polychaeta). *Bulletin of Marine Science*, 67, 421-432.
- Pauly, D; Munro, J L. (1984). Once more on the comparison of growth in fish and invertebrates. *Fishbyte*, 2, 21.
- Pauly, D; Moreau, J; Abad, N. (1995). Comparison of age structure and length-converted catch curves of brown trout *Salmo trutta* in two French rivers. *Fisheries Research*, 22, 197-204.
- Rios, EC. (1994). *Seashells of Brazil*. Rio Grande, 368p., Fundação Universidade de Rio Grande.
- Ricker, WE. (1975). Computation and interpretation of biological statistics of fish populations. *Bulletin of the Fisheries Research Board of Canada*, 191, 1-382.
- Sardá, R; Foreman, K; Valiela, I. (1995). Macroinfauna of a southern New England salt marsh : seasonal dynamics and production. *Marine Biology*, 121, 431-445.

- Sola, J C. (1996). Population dynamics, reproduction, growth, and secondary production of the mud-snail *Hydrobia ulvae* (Pennant). *Journal of Experimental Marine Biology and Ecology*, 205, 49–62.
- Tararam, AS; Wakabara, Y. (1987). Benthic fauna living on *Spartina alterniflora* of Cananéia estuarine region (25 02'S – 47 56'W). *Boletim do Instituto Oceanográfico*, 35, 103–113.
- Tumbiolo, ML; Downing, JA. (1994). An empirical model for the prediction of secondary production in marine benthic invertebrate populations. *Marine Ecology*, 114, 165-174.
- Underwood, AJ. (1984). Microalgal food and the growth of the intertidal gastropods *Nerita atramentosa* Reeve and *Bembicium nanum* (Lamarck) at four heights on a shore. *Journal Experimental Marine Biology and Ecology*, 79, 277-291.
- Virnstien, RW. (1980). Predation on estuarine infauna: response patterns of component species. *Estuaries*, 2, 69-86.
- Warmke, G; Abbott, RT. (1962). *Caribbean Seashells*. New York, 348p., Livingston Publ.
- Yerro, AB. (1984). *Rissoa parva* (da Costa), *Barleeia unifasciata* (Montagu) y *Bittium reticulatum* (da Costa) (Mollusca: Gastropoda): Estudio de la segregación de tres estrategias de crecimiento, dinámica y producción sobre *Halopteris scoparia* (L.) Sauv. Ph.D. Thesis, Universidad del País Vasco.
- Zar, J.H. (1996). *Biostatistical Analysis*. New Jersey, 718p., Prentice-Hall.

REPRODUCTION AND LIFE HISTORY IN THE TWO LAND SNAILS *MONACHA CARTUSIANA* (MÜLLER) AND *EOBANIA VERMICULATA* (MÜLLER) (HELICIDAE: MOLLUSCA) IN THE LABORATORY

M. I. Mohamed and R. F. Ali

Department of Zoology and Agricultural Nematology,
Faculty of Agriculture Cairo University; Giza, Egypt

ABSTRACT

Mating, oviposition, post-oviposition period, development, and generation period of the two land snail species *Monacha cartusiana* (Müller) and *Eobania vermiculata* (Müller) were studied in the laboratory in Egypt during the two seasons of 2003/4 and 2004/5. Data revealed that mating is essential for both snail species to lay eggs, thus it occurs when snails reached their sexual maturity (from adulthood till mating) during a period averaged 190.3 and 228.6 days for *M. cartusiana* and 231.6 and 568.3 days for *E. vermiculata* after adulthood in the two different seasons respectively. Pre-oviposition period (from mating till laying first egg) lasted an average of 2.7 and 8.2 days for *M. cartusiana* and 3.5 and 9.2 days for *E. vermiculata* during the two seasons respectively.

Oviposition period (from first laying egg till last one) averaged 22.5 and 64.1 days during which the snail of *M. cartusiana* deposited an average of 63.0 and 177.5 eggs during the two seasons respectively. On the other hand this period averaged 15.2 and 24.9 days for *E. vermiculata* during which the snail individual deposited and total average of 89.6 and 162.0 eggs during 2003/4 and 2004/5 respectively. While, post-oviposition period (from last egg till death) lasted 142.4 and 71.9 days for first snail species and 372.5 and 660.1 days for the second one during the same seasons respectively. The generation period (from egg to egg) extended an average of 369.8 and 402.6 and 481.7 and 868.7 days for both species during the same seasons respectively.

Keywords: *Monacha cartusiana* – *Eobania vermiculata* – Mating – Oviposition– Generation period – Sexual maturity.

INTRODUCTION

Land molluscs can cause considerable damage to field and horticultural crops in the world. Runham and Hunter (1970) reported that gastropod pests fed on cereals, potatoes,

vegetables, lettuce, maize, carrots, beetroot, clover, cabbage, turnip, as well as horticultural crops and ornamental plants. Management and control of such pests requires basic information on their life histories and habits.

In Egypt, the snails *Monacha cartusiana* and *Eobania vermiculata* are two the most important pest species, which the first infested 38 different host plants of field crops (7), vegetables (11), fruit trees (4), ornamental plants (5) and weeds (11) ; while the second species was found on 17 ornamental host plants and two fruit trees at different localities.

The main objective of this investigation was to spot the more light about oviposition, life cycle, life span, and reproductive potentiality of these pests in the Egyptian lands.

MATERIAL AND METHODS

50 adult individuals of each the glassy clover snail *M. cartusiana* and the brown garden snail *E. vermiculata* were collected by hand from infested host plants during their active period in November 2003 for rearing during season 2003/4 and repeated again in November 2004 for season 2004/5 and both were observed till death. Members of each species were kept in plastic boxes 15 x 7 x 11 cm and 27 x 27 x 25 cm contained moist soil to a depth 8 – 10 cm and provided with fresh lettuce leaves *Lactuca sativa* L. as a main source of food; then covered with muslin cloth fixed with rubber bands to prevent snails from escaping.

These cultural boxes were examined daily; fresh food and moisture were supplied as required, and the soil was searched for new clutches of eggs. Newly deposited clutches were removed then placed in prepared pots (13 x 10 cm) with moist soil.

They were observed daily till hatching to determine the incubation period as well as hatchability. Juveniles of both species were placed singly each in plastic cups (10 x 6 cm) filled with moist clay soil of about five centimeters and provided with fresh lettuce leaf disc then covered to avoid snail escaping and examined daily; fresh food and moisture were added as required until reached maturity. Mortality ratio among these juveniles averaged 14% for *M. cartusiana* and 18% *E. vermiculata* before reaching adulthood.

Adult snails of each reared species were paired in prepared plastic pots (13 x 10 cm) and observed several times daily till mating occurs to determine their sexual maturity period and mating behavior. After mating every pair was separated and each individual placed in a new prepared plastic pot to determine the pre-oviposition period, oviposition period, number of clutches per individual, clutch size, as well as post-oviposition period.

RESULTS

A. Mating

Both these species are hermaphrodites, and, as is normal for Helicids, self-fertilization does not occur. Mating usually took place when individuals reach their sexual maturity during a period which differed according to season and species. This period averaged 190.3 and 228.6 days for *M. cartusiana* during season 2003/4 and 2004/5 respectively, while it averaged 231.6 and 568.3 days for *E. vermiculata* during the same seasons respectively after adulthood

was attained (Tables 1 and 2). These results obtained from 10 and 20 and 13 and 12 pairs were involved in each set of observations for the two species in the same seasons respectively. (Figure 1)

Table 1. Duration of different stages of *M. cartusiana* during two seasons

Season	Average period in days $\pm$ SD							
	Incubation period	Juveniles	Life cycle	Sexual maturity	Pre - Oviposition	Oviposition	Post - Oviposition	Life span
Average $\pm$ SD for 10 snail/pair 2003/4	19.3 $\pm$ 3.6	185.8 $\pm$ 61.8	204.1 $\pm$ 59.5	190.3 $\pm$ 35.3	2.7 $\pm$ 1.4	22.5 $\pm$ 23.8	142.4 $\pm$ 105.6	497.7 $\pm$ 69.6
Range	12-25	124-319	136-344	153-237	1-5	1-76	20-362	376-708
Temp. $\pm$ SD	18.1 $\pm$ 1.6°C	19.8 $\pm$ 1.8°C	18.6 $\pm$ 0.9°C	19.2 $\pm$ 1.8°C	17.4 $\pm$ 0.3°C	17.9 $\pm$ 1.2°C	18.2 $\pm$ 0.7°C	21.8 $\pm$ 3.4°C
Average $\pm$ SD for 20 snail/pair 2004/5	18.1 $\pm$ 3.7	141.3 $\pm$ 12.1	156.5 $\pm$ 15.3	228.6 $\pm$ 12.4	8.2 $\pm$ 4.6	64.1 $\pm$ 25.6	71.9 $\pm$ 62.8	529.4 $\pm$ 78.6
Range	13-29	112-154	125-183	216-259	2-29	9-103	10-225	376-760
Temp. $\pm$ SD	18.7 $\pm$ 0.9°C	19.2 $\pm$ 1.3°C	18.8 $\pm$ 1.1°C	20.2 $\pm$ 2.1°C	19.8 $\pm$ 0.8°C	19.8 $\pm$ 0.8°C	20.1 $\pm$ 1.3°C	22.6 $\pm$ 3.7°C

During the reproductive season (mainly from mid November till mid January); mating in both species usually occurs at night and its timing differed according to species, in *M. cartusiana* from 5.0 p.m. till 11.0 p.m. with a maximum during the period from 8.0 p.m. to 10.0 p.m., while in *E. vermiculata*, mating usually occurred between 10.0p.m. and 3.0 a.m.

During mating process two snail individuals faced each other and attached together strongly from the fleshy parts. Copulations ranged from 90 to 225 minutes with an average of 143.0 ± 48.5 ($\pm$ SD) minutes for *M. cartusiana*, while it ranged from 300 to 420 minutes with an average of 325.5 ± 43.0 minutes for *E. vermiculata*. After this period, disengagement takes place gradually over a period of averaging 22.5 ± 1.6 minutes for *M. cartusiana* and 35.0 ± 4.1 minutes for *E. vermiculata*.

Table 2. Duration of different stages of *E. vermiculata* under laboratory conditions

Season	Average period in days ± SD							
	Incubation period	Juveniles	Life cycle	Sexual maturity	Pre-oviposition	oviposition	Post-oviposition	Life span
Average ±SD for 13 snail/pair 2003/4	21.0 ±3.8	185.3 ±86.6	210.1 ±84.1	231.6 ±106.6	3.5 ±1.9	15.2 ±8.3	372.5 ±113.3	920 ±161.3
Range	11-25	77-371	104-396	111-445	1-6	1-26	139-550	631-1367
Temp.± SD	19.6± 1.7°C	20.6± 1.4°C	20.8± 1.9°C	19.3± 1.2°C	17.4± 0.2°C	18.2± 1.7°C	23.2± 2.7°C	21.6± 2.4°C
Average ±SD for 12 snail/pair 2004/5	21.1 ±4.7	239.4 ±127.1	293.7 ±147.0	568.3 ±284	9.2 ±8.9	24.9 ±23.3	660.1 ±272.7	1532.4 ±490
Range	11-30	101-597	122-620	138-1074	1-24	1-57	300-1066	603-2760
Temp.± SD	18.9± 0.8°C	19.8± 1.8°C	21.0± 1.4°C	20.6± 1.9°C	20.8± 0.5°C	20.6± 1.1°C	23.7± 2.9°C	22.7± 3.4°C



Figure 1. Mating process of *M. cartusiana*.

B. Oviposition

During the present study it was observed that unmated snails of *M. cartusiana* and *E. vermiculata* did not lay any eggs. Numbers of unmated *M. cartusiana* were 20 and 22 snail, while *E. vermiculata* were 24 and 20 snail for 2003/4 and 2004/5 respectively. Also, it was noticed that both mated snails 3 and 20 snail/pair of *M. cartusiana* and 8 and 6 snail/pair of *E. vermiculata* laid eggs during the two seasons respectively. On the other hand it was found that only one of both mated snails deposited eggs ($N. = 5$ and 0 snail/pair for *M. cartusiana* and 3 and 4 snail/pair for *E. vermiculata* during the same seasons respectively). Sometimes both mated snails did not lay any eggs and this might be due to their failed in copulation ($N. = 2$ and 0, 2 and 2 for both species during the same period respectively). (Tables 3 and 4). The pre-oviposition period (between mating and laying) differed according to species and season (Tables 1 and 2). It averaged 2.7 and 8.2 days from mating for *M. cartusiana* and 3.5 and 9.2 days for *E. vermiculata* for 2003/4 and 2004/5 respectively.

Table 3. Duration of *M. cartusiana* life span in two seasons

Season	Average life span period in days $\pm$ SD		
	Mated snails		Unmated
	Laid	Non laid	
2003 / 2004	497.7 $\pm$ 69.6	506.1 $\pm$ 122.7	541.1 $\pm$ 111.7
Range	376 – 708	305 - 652	335 - 761
No. of snails	11	9	20
2004 / 2005	529.4 $\pm$ 78.6	0.0	562.4 $\pm$ 106.9
Range	376 - 760	0.0	330 - 680
No. of snails	40	0	22

Table 4. Duration of *E. vermiculata* life span in two seasons

Season	Average life span period in days $\pm$ SD		
	Mated snails		Unmated snails
	Laid	Non laid	
2003 / 2004	920.0 $\pm$ 161.3	434.9 $\pm$ 106.6	605.6 $\pm$ 156.1
Range	631 – 1367	334 – 685	375 – 863
No. of snails	20	6	24
2004 / 2005	1532.4 $\pm$ 490	508.7 $\pm$ 97.5	702.8 $\pm$ 159.9
Range	603 – 2760	334 – 685	375 - 898
No. of snails	19	5	20

Snails preferred to deposit eggs in protected places; thus, *M. cartusiana* made small pits or holes one to three centimeters deep in the soil, while *E. vermiculata* dug a circular hole or chamber five to six centimeters deep; then each species laid its eggs singly at one time and adhere to one another in the same place forming an egg mass which so called batch, or cluster, or clutch, as well as sometimes laid its eggs singly scattered in the soil each one in small hole then covered with soil. *M. cartusiana* individuals spent an average time of 150.0 ± 5.4 minutes for depositing one clutch (which averaged 17.0 ± 1.2 eggs), while those of *E. vermiculata* took 362.5 ± 46.0 minutes on average depositing an average clutch of 55.3 ± 18.3 eggs (these observations from five individuals of each species and $\pm$ means SD).

M. cartusiana deposited an average of 5.0 ± 4.5 and 15.0 ± 7.1 clutches during its oviposition period which averaged 22.5 ± 23.8 and 64.1 ± 25.6 days in the two seasons 2003/4 and 2004/5. The rate of deposited individual clutches per day averaged 1.2 ± 0.7 and 1.2 ± 0.4 and the period elapsed between each deposited clutch to another averaged 5.6 ± 8.5 and 4.5 ± 4.7 days during the same seasons respectively. The clutch size averaged 11.4 ± 9.3 and 10.4 ± 8.3 eggs. Thus, the total number of deposited eggs per one individual of this species during its oviposition period averaged 63.0 ± 48.4 and 177.5 ± 103.5 eggs at the same seasons respectively. The eggs are white, with a smooth surface and spherical shape (mean diameter 1.6 ± 1.1 mm). 50 eggs weighed 0.08 gm.

E. vermiculata, during its oviposition period which averaged 15.2 ± 8.3 and 24.9 ± 23.3 days deposited an average of 2.2 ± 1.2 and 2.3 ± 1.4 clutches in the first and second seasons respectively. The rate of deposited clutches per day was one clutch. The period from each deposited clutch to another averaged 11.3 ± 4.6 and 16.3 ± 11.9 days during the same seasons respectively. The clutch size averaged 36.9 ± 20.0 and 44.9 ± 38.4 eggs. Thus the total number of deposited eggs by one individual of this species during its oviposition period averaged 89.6 ± 36.2 and 162.0 ± 30.1 eggs in the same two seasons respectively. Eggs of this species are white, with a smooth surface, and rounded to ovate in shape. Means of length and greatest width were 3.1 and 3.0 mm respectively and a clutch size of an average 40 eggs weighed 0.7 ± 0.5 gm.

C. Post-Oviposition

The post-oviposition period as calculated from the last deposited egg till snail individual death, followed the same trend as it differed according to species and season. This period averaged 142.4 and 72.0 days for *M. cartusiana* during the seasons of 2003/4 and 2004/5 respectively (Tables 1 and 2). While, for *E. vermiculata* it averaged 372.5 and 660.1 days during the same two seasons respectively.

D. Development

Length of incubation period differed according to species and seasons (Tables 1 and 2) as it averaged 19.3 and 18.1 days for *M. cartusiana* during seasons 2003/4 and 2004/5 respectively, while it averaged 21.0 days in *E. vermiculata* during both seasons. Hatching success also differed according to species averaging 74.0 ± 27.8 and 77.8 ± 24.0 % for *M.*

cartusiana and 68.1 ± 31.0 and 71.2 ± 31.9 for *E. vermiculata* during the same two seasons respectively.

The maximum shell diameter of newly hatching juveniles was 1.7 ± 0.2 mm for *M. cartusiana* and 3.03 ± 0.1 mm for *E. vermiculata*. After 22 weeks (154 days) from hatching, *M. cartusiana* individuals had achieved a mean maximum shell diameter of 11.8 ± 0.4 mm, while those of *E. vermiculata* achieved a mean of 26.1 ± 0.0 mm after 30 weeks. Mean times from hatching to adulthood (juvenile's duration) were 185.8 and 141.3 days for *M. cartusiana* and 210.0 and 239.4 days for *E. vermiculata* during the two seasons respectively (Tables 1 and 2).

Thus the duration of life cycle from egg to adult (incubation period as well as juveniles) for *M. cartusiana* averaged 204.1 and 156.5 days, while it averaged 210.1 and 293.7 days in *E. vermiculata* during the same two seasons respectively (Tables 1 and 2). Life span (from egg till death) of both species differs according to mating, oviposition and season (Tables 3 and 4). In *M. cartusiana*, life span of unmated snails was longer (541.1 and 562.4 days) than mated in the two seasons. On the other hand, in mated snails this period differed according to oviposition; it averaged 497.7 days for laying (life cycle, sexual maturity, pre-oviposition, oviposition and post-oviposition period) and 506.1 days for non-laying snails (life cycle, sexual maturity till death) in season 2003/4. In the next season all mated snails deposited eggs, thus its life span averaged 529.4 days. For *E. vermiculata*, mating and oviposition prolonged its life span period in both seasons, followed by unmated snails while the lowest period was recorded in non-laying mated snails (Table 4).

E. Generation Period

Generation period (from egg to egg) was calculated only for laying snails, and it includes; life cycle, sexual maturity and pre-oviposition periods. Thus it averaged 369.8 ± 7.0 and 402.6 ± 30.2 days for *M. cartusiana* and 481.7 ± 140.7 and 868.7 ± 339.6 days for *E. vermiculata* during the seasons 2003/4 and 2004/5 respectively.

DISCUSSION

During the present study it was observed that copulation is essential for oviposition as unmated snails of both species *M. cartusiana* and *E. vermiculata* did not lay any eggs. Also, it was noticed that both mated snails or only one from both mated deposited eggs and sometimes both mated individuals could not lay any eggs and this may be due to their copulation failed. These results are in accordance with (Takeda, 1983), (Bride and Gomot, 1991) and (Bride *et al*, 1991), who mentioned that egg production in several species of stylommatophoran gastropods is stimulated by mating behavior. On the other hand, Capinera (2001) recorded that both mated individuals of *Helix aspersa* Müller deposited eggs.

Laboratory observations indicated that, before depositing eggs, the snail of both species required after mating a pre-oviposition period which differs from individual to another according to season which averaged 2.7 and 8.2 days and 3.5 and 9.2 days for *M. cartusiana* and *E. vermiculata* during two seasons respectively and these accepted with those mentioned

by (Heller, 1982) who recorded that *Thepa pisana* (Müller) laid its eggs from 9.0 to 14.0 days after copulation; (Baur and Baur, 1992) mentioned that *Arianta arbustorum* L. snails which had copulated deposited their first batch after 10.3 ± 1.5 days, and (Capinera, 2001) who stated that the brown garden snail *H. aspersa* deposited eggs beginning 3 – 6 days after mating. While, (Stringer *et al.*, 2003) mentioned that this period reaches 140 days in *Paryphanta busbyi watti*.

Both individuals of *M. cartusiana* and *E. vermiculata* preferred to laid their eggs in protected places in batches or scattered in the soil each in separate hole and these accepted with those mentioned by (Baur, 1990) that *A. arbustorum* usually lays its eggs either in batches in small holes in the soil or singly on the soil surface, (South, 1992) who indicated that, most terrestrial gastropod species are oviparous and for oviposition some of them utilize natural holes or crevices in the soil or under stones and pieces of wood. On the other hand, (Baur, 1994) mentioned that *Zonitoides arboreus* (Say) lays single eggs scattered in forest litter.

Data indicated that, the total number of deposited eggs per individual differed according to species and season. Thus, the total number was 63.0 and 177.5 eggs during an oviposition period of 22.5 and 64.1 days for *M. cartusiana* during seasons 2003/4 and 2004/5 respectively, while those of *E. vermiculata* averaged 89.6 and 162.5 eggs during an average period of 15.2 and 24.9 days in the same seasons respectively. While, (Staikou and Lazaridou, 1990) mentioned that the mean number of eggs laid per snail of *M. cartusiana* was 32.67 ± 10.82 . On the other hand, (Capinera, 2001) recorded that, the number of eggs deposited by *H. aspersa* at one time varies from about 30 – 120, averaging 86 and the total fecundity is estimated at 400 – 450 eggs annually.

Life cycle or maturity (including incubation period and juveniles) differs also according to species and season as it averaged 204.1 and 156.5 days for *M. cartusiana* and 210.0 and 293.7 days for *E. vermiculata* during the two seasons respectively. Staikou and Lazaridou (1990) decided that, 14% of *M. cartusiana* individuals reached maturity one year after hatching, while the majority reached maturity and laid eggs two years after hatching and all snails died after the reproductive period.

Sexual maturity period (including life cycle till mating) of both species averaged 190.3 and 228.6 days and 231.6 and 568.3 days after adulthood for *M. cartusiana* and *E. vermiculata* during the two seasons respectively; while, (Staikou and Lazaridou, 1990) found that, the sexual maturity of *M. cartusiana* was normally attained two years after hatching and (Stringer *et al.*, 2003) mentioned that mating occurs at 406.2 ± 63.8 days after development from juvenile to adult in *Paryphanta busbyi watti*.

The life span period (from hatching till death) averaged 497.7 and 529.4 days and 920 and 1532.7 days for *M. cartusiana* and *E. vermiculata* during the same two seasons respectively and this agree with those by (Heller, 1990) who mentioned that, life span is variable being annual in one habitat but stretching over several years in a different habitat with the range from several months to 19 years during his study on 75 species belonging to 57 genera and 30 families.

REFERENCES

- Baur, B. 1990. Seasonal changes in clutch size, egg size and mode of oviposition in *Arianta arbustorum* L. (Gastropoda) from Alpine populations. *Zoologisch Anzeiger*. 225: 253 – 264.
- Baur, B. 1994. Parental care in terrestrial gastropods. *Experientia* 50, 5 – 14.
- Brid, J. and L. GOMOT. 1991. In vitro demonstration of direct stimulation of the helix aspersa albumen gland galactogen synthesis by extracts from the male accessory glands of the genital tract. C. R. *Academic Science Paris Ser.*, 111 (313): 565 – 572.
- Brid, J. and L. Gomot, and A. S. M. Saleuddin. 1991. Mating and 20-hydroxyecdysone cause in increased galactogen synthesis in the albumen gland explants of helix aspersa (Mollusca). *Comparative Biochemistry and Physiology*, 98 (B): 369 – 373.
- Capinera, J. L. 2001. *Handbook of vegetable pests*. Academic Press, New York, London, 729 pp.
- Heller, J. 1982. Natural history of *Thepa pisana* in Israel (Pulmonata: Helicidae). *Journal Zoology London*, 196: 475 – 487.
- Heller, J. 1990. Longevity in molluscs. *Malacologica*, 31 (2): 259 – 295.
- Lazaridou-Dimitriadou, M. and M. E. Kattoulas. 1991. Energy flux in a natural population of the land snail *Eobania vermiculate* (Müller) (Gastropoda, Pulmonata: Stylommatophora) in Greece. *Canad. J. Zool.*, 69 (4): 881 – 891.
- Runham, N. W. and P. J. Hunter. 1970. *Terrestrial slugs*. Hutchinson (Publishers) LTD. London, 184 pp.
- South, A. 1992. *Terrestrial slugs*. Chapman and hall, London.
- Staikou, A. and M. Lazaridou-Dimitriadou. 1990. Aspects of life cycle, population dynamics, growth, and secondary production of the snail *Monacha cartusiana* (Müller, 1774) (Gastropoda: Pulmonata) in Greece. *Malacologia*, 31 (2): 353 – 362.
- Stringer, A. N. Ian; S. M. Bassett; M. J. Mclean; J. McCartne and G.R. Parrish. 2003. Biology and conservation of the rare Newzealand land snail *Paryphanta busbyi watti* (Mollusca, Pulmonata). *Invertebrate Biology*, 122 (3): 241 – 251.
- Takeda, N. 1983. Endocrine regulation of reproduction in the snail *Euhadre peliomphala*. *Molluscan Neuroen.*, J. Lever and H. H. Boer, 106 – 111.

EFFECT OF SUBLETHAL CONCENTRATION OF ARSENIC ON HEMOCYTE DENSITY IN EDIBLE CRAB, *Scylla serrata*

Sanjib Saha, Mitali Ray and Sajal Ray*

Aquatic Toxicology Laboratory,
Department of Zoology, University of Calcutta.
35 Ballygunge Circular Road, Kolkata – 700019, India

ABSTRACT

Sundarbans biosphere reserve is a unique ecosystem and characteristic of diverse types of habitats ranging from mudflat to sandy beaches. Nutritionally, economically and ecologically important estuarine edible crab, *Scylla serrata* (Arthropoda: Crustacea: Decapoda) occupy diverse habitats and their survival is dependent on multidimensional immune responses mostly offered by hemocytes – the chief immunoeffector cells of hemolymph. The circulating hemocyte number is a stress indicator and hemocyte counts may be a valuable tool in monitoring the health status in biounsafe environment in *S. serrata*. The enumeration of total and differential hemocyte has been enumerated under the exposure of 1, 2 and 3 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15 and 30 days in controlled laboratory condition. In *S. serrata* diverse subpopulations of hemocytes were identified as hyalinocytes, semigranulocytes and granulocytes. Alteration in hemocyte counts and density of hemocytes were recorded under 1, 2 and 3 ppm of arsenic in static water environment. Shift in THC and DHC recovered partially in arsenic exposed crabs maintained in arsenic free water up to 30 days. Exposure to 1 ppm of sodium arsenite for 30 days yielded a highest THC as $312.9 \pm 3.6 \times 10^5$ cells / ml against control value of $47.319 \pm 4.8 \times 10^5$ cells / ml. Relatively high THC in the crab maintained in toxin free water for 30 days of span indicates a persistence of cellular stress induced by arsenic exposure. A highest depression in hyalinocyte density was recorded under the exposure of 1 ppm of arsenite for 30 days as 1.17% against control value as 7.47%. Similarly decrease of granulocyte was recorded as 16.21% in 30 days of exposure against control value of 27.24%. Elevation of semigranulocyte was recorded as 82.62% against the control value of 65.29%. Study of possible restoration of DHC was carried out by maintaining the animals in toxin free water for 30 days. All groups of treated animals excepting the groups exposed to 2 ppm for 30 days and 3 ppm for 30 days recovered the

* Corresponding author: E- mail: raysnailmail@rediffmail.com; Telephone – 91(033)24229800; Fax no. – 91(033)24614849.

DHC of the control. Depression in hyalinocyte and semigranulocyte density was recorded under the exposure of 3 ppm of arsenite for 30 days against control value. Arsenic induced alteration in hemocyte density indicated a state of immunological stress in estuarine crab. Data is suggestive of cellular stress which may lead to decline in endemic population of *S. serrata* in arsenic affected districts of West Bengal.

Keywords: *Scylla serrata*, Hemocyte, Sodium arsenite.

INTRODUCTION

Sundarbans biosphere reserves ('World Heritage' in 1987 by IUCN and 'Biosphere Reserve' in 1989 by UNESCO) of West Bengal, India supports a wide diversity of crabs (Chaudhuri and Choudhury, 1994). Sundarbans mangrove swamp is an unique ecosystem which is occupied a varied range of flora and fauna. Sundarbans delta is one of the largest mangrove forest which is characteristics of diverse habitat including micro and macro. Tidal submergence and periodic inflow of nutrients make this ecosystem dynamic in relation to physiological status of the animals. *Scylla serrata* or estuarine mud crab is an important inhabitant of this ecosystem (Ali *et al.* 2004). This animal provides an important item of food for human and other consumers. Sundarbans delta receives toxins of varied chemistry yet to be characterized in depth (Ghosh *et al.*, 2008). Due to continuous contamination of this estuarine ecosystem inhabitation of Sundarbans are in continuous exposure to different xenobiotics including arsenic (Pal *et al.*, 2002; Sarkar *et al.*, 2004). Arsenic is an important toxic metalloid is potentially capable of creating physiological disturbances both at cellular and sub-cellular levels (Krishnaja *et al.*, 1987; Shibu Vardhanan and Radhakrishnan, 2002). Sublethal toxicity of arsenic in mud crab is a poorly studied area of research which demands special attention from toxicologist and ecologist (Gomez-Camirero *et al.*, 2001). Hemocytes are cellular component of the blood or hemolymph and these morphologically discrete cells are the chief component of crustacean immune system (Saha and Ray, 2006). Under the exposure of toxins and parasites hemocytes of invertebrates are capable of eliciting specific immunological response (Saha *et al.*, 2007; Chakraborty *et al.*, 2008, Mukherjee *et al.*, 2008). Phagocytosis of non-self particulates, recognition of non-self surface, cell-cell aggregation and generation of cytotoxic molecules are the distinct responses offered by them (Saha *et al.*, 2008).

In the present study, we attempt to examine alteration of total, differential hemocyte counts (THCs and DHCs) and its restoration of *S. serrata* in order to characterize the immune status under exposure of sodium arsenite in controlled laboratory condition and provide an information base in understanding the degree of contamination of aquatic environment by various xenobiotics and pathogens.

MATERIALS AND METHODS

1. Collection and Transportation of *S. serrata*

Live adult specimens of *S. serrata* weighing 65-75 gm were collected from selected habitats of south 24 Parganas of West Bengal, which are relatively free from arsenic

contamination. Length and breadth of the carapace of experimental animals were 6 ± 0.5 cm and 9 ± 0.5 cm respectively. Live crabs were transported to the laboratory in rectangular plastic boxes (Saha and Ray, 2006).

2. Acclimatization and Maintenance of *S. serrata*

Live animals were maintained in arsenic free estuarine water and fed with fresh flesh of molluscs (*Bellamya bengalensis*) once a day. The water of glass aquaria was replenished in every 24 hours to avoid residual toxicity. Animals were acclimatized in laboratory conditions for 6-8 days prior to experimentation. During experiments experimental subjects received uniform ration of illuminated and temperature of water ranged from 25 - 30 °C (Heasman and Fielder, 1983).

3. Treatment with Sodium Arsenite

Measured amount of sodium arsenite (E. Merck, Germany; 99% pure; CAS number 7784-46-5) were dissolved in different experimental doses. During treatment mouth of test concentration (Borosilicate glass beaker) were partially covered by glass lids to prevent atmospheric desiccation. Height of the water column never exceeded a depth of two inches both for control and treatment states. All the containers were fitted with electrically operated aerator to facilitate supply of oxygen. Determination of median lethal concentration (LC₅₀) of sodium arsenite was done by 'Behrens-Karber method' after Klassen (1991).

4. Rationale of Selecting the Experimental Concentrations

In static water environment median lethal concentration (LC₅₀) of sodium arsenite is determined to be 15 ppm and 10 ppm in adult and juvenile crabs respectively for 96 hours of span. In this present study, all the experimental concentrations of sodium arsenite were selected to be less than 50% of LC₅₀ value. According to Das and Roy Chowdhury (2006) concentration of arsenic in aquatic ecosystem of W.B ranges from 0.5 – 3.2 ppm in different seasons. Dynamics of concentration of dissolved arsenic is due to several environmental factors i.e. desiccation, dilution and natural availability. All the experimental concentrations of sodium arsenite were less than the highest reported natural concentration of arsenic.

5. Collection of Hemolymph

Fresh hemolymph of *S. serrata* was collected aseptically from the base of one of the second walking leg using a sterile syringe with a 23-gauge needle. Collected blood was stored in prechilled sterile glass vials containing 2 % EDTA as anticoagulant to avoid cells aggregation and the volume of collected blood of each animal did not exceed 0.5 ml at one extraction (Yildiz and Atar, 2002). Suspended hemocytes were sedimented by cold

centrifugation (Hermle, Germany) of the hemolymph at 3000 rpm for 5 minutes. Cell density was adjusted with uniform volume of sterile phosphate buffer saline (PBS) as and when necessary. Free and live hemocytes in suspension were observed and screened under inverted microscope (Axiovert, Zeiss, Germany).

6. Enumeration

Total count of hemocyte was carried out using improved Neubauer hemocytometer under light microscope (Axiostar, Zeiss, Germany) after Saha and Ray, 2007. Generated data was an average of at least five observations for each experiment. Live hemocyte suspension were smeared over glass slide (Blue star, India) and were semi dried. Smear cells were subjected to treat with methanol for 2-3 min for fixation. Unstained fix cells were examined under phase contrast microscope (Axiovert, Zeiss, Germany) prior to stain. The number of hyalinocytes, semigranulocytes and granulocytes (differential hemocyte count, DHC) were estimated using bright field after fixing and staining hemocytes with Wright stain (SRL, India) throughout the study and taken digital photograph by camera-attached microscope (Axiostar Zeiss, Germany). Number of each cell type was expressed in percentage (Saha and Ray, 2006). To screened possible recovery of hemocyte population, all sets of treated animals were maintained in arsenic free water upto 30 days. Enumeration of restored density of hemocyte was estimated under bright field similar as above.

8. Statistical Analyses

All experiments were repeated for three times and values are expressed as mean $\pm$ SD for THC and percentage (%) for DHC estimation. The data are compared in paired Fisher t-test and symbols indicate values that are significantly different from controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Median lethal concentration for 96 hours of sodium arsenite in adult *S. serrata* was 15 ppm in static water environment. Mean total hemocyte count (THC) was $47.31 \pm 4.8 \times 10^5$ cells / ml (Figure 1). Alteration in hemocyte density was recorded against 1, 2 and 3 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15 and 30 days. The lowest and highest THC was accounted for the specimen exposed to 1 ppm of sodium arsenite for 3 and 30 days respectively (Figure 2). Restoration of THC of post treated (1, 2 and 3 ppm for 4, 15 and 30 days) *S. serrata* were maintained in arsenic free water for 5, 10, 20 and 30 days (Table 1). 100 % restoration of THC was not screened in post-treated crabs even after maintenance in arsenic free water up to 30 days. Maximum and minimum restoration in THC was accounted against 1 ppm / 4 days and 3 ppm / 30 days respectively (Table 1). In control the most abundant type of hemocyte was semigranulocytes consisting 65.29%, where as hyalinocytes and granulocytes consisted 7.47% and 27.24% respectively (Figure 2).

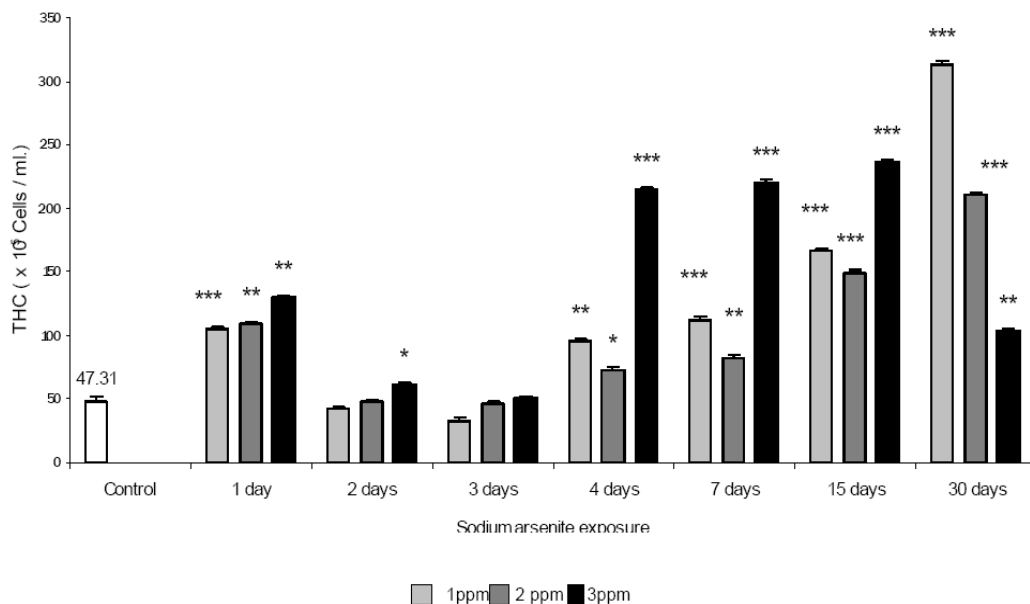


Figure 1. Alteration of total hemocyte count (THC) of adult *S. serrata* under exposure of 1, 2, 3 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15 and 30 days. Data expressed as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 5$).

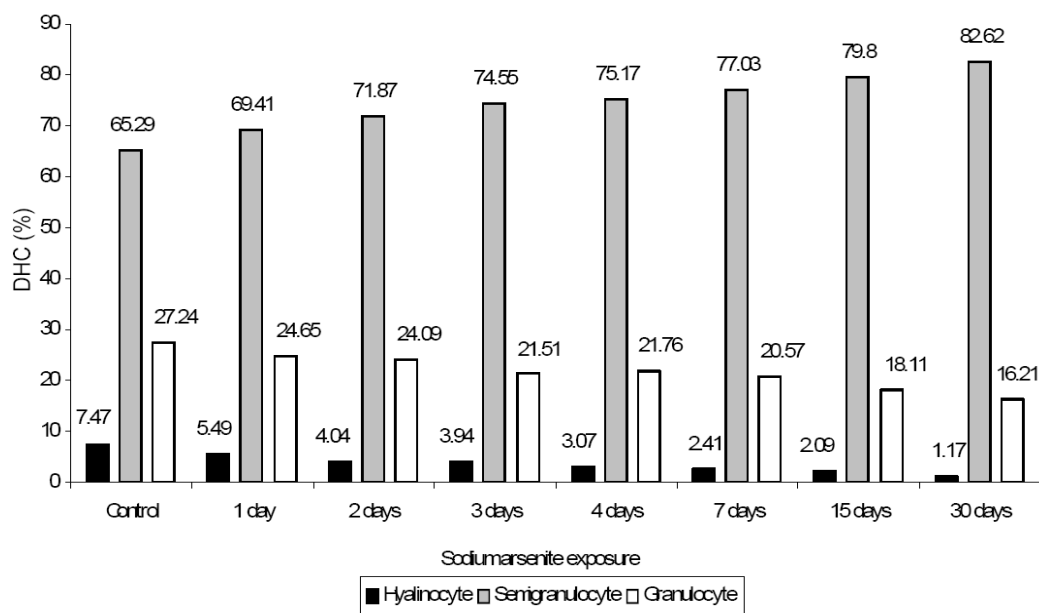


Figure 2. Alteration of differential hemocyte count (DHC) of *S. serrata* under exposure of 1 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15, 30 days. Data expressed as percentage ($n = 5$).

Table 1. Restoration of total hemocyte count (THC) of post treated (1, 2, 3 ppm sodium arsenite for 4, 15 and 30 days) *S. serrata* maintaining in arsenic free water for 30 days. Data expressed as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, * $P < 0.001$ (n = 5)**

Control THC (x 10 ⁵ cells/ml)	THC in post-treated <i>S. serrata</i> (x10 ⁵ cells / ml)		THC of <i>S. serrata</i> maintained in arsenic free water (x10 ⁵ cells / ml)			
	ppm / Days	Treated THC	Day 5	Day 10	Day 20	Day 30
47.31 $\pm$ 4.8	1 ppm / 4 days	95.04 $\pm$ 1.83**	91.4 $\pm$ 3.9**	80.60 $\pm$ 3.6**	66.6 $\pm$ 4.1*	47.98 $\pm$ 1.49
	1 ppm / 15 days	166.5 $\pm$ 2.3***	112 $\pm$ 1.83***	95.04 $\pm$ $\pm$ 1.63**	72.96 $\pm$ 1.72*	51.68 $\pm$ 2.75
	1 ppm / 30 days	312.9 $\pm$ 3.6***	248 $\pm$ 4.8***	202 $\pm$ 5.1***	120.2 $\pm$ 3.7***	72.1 $\pm$ 3.2*
	2 ppm / 4 days	72.96 $\pm$ 1.4*	70.1 $\pm$ 1.6*	68.8 $\pm$ 3.9*	57.2 $\pm$ 3.6*	48.12 $\pm$ 2.9
	2 ppm / 15 days	149.2 $\pm$ 2.2***	129.6 $\pm$ 1.92***	114.2 $\pm$ 1.7***	95.04 $\pm$ 1.83***	62.97 $\pm$ 1.63*
	2 ppm / 30 days	210 $\pm$ 2.3***	157.5 $\pm$ 1.49***	127.2 $\pm$ 2.1***	105.6 $\pm$ 2.2***	78.2 $\pm$ 3.5*
	3 ppm / 4 days	214.4 $\pm$ 1.8***	189.63 $\pm$ 2.1***	156.6 $\pm$ 1.59***	88.21 $\pm$ 1.89**	49.26 $\pm$ 2.6
	3 ppm / 15 days	236 $\pm$ 2.4***	178.1 $\pm$ 2.3***	166.5 $\pm$ 5.2***	138.9 $\pm$ 1.49***	68.3 $\pm$ 2.2**
	3 ppm / 30 days	104.2 $\pm$ 2.5**	95.6 $\pm$ 1.89**	92.3 $\pm$ 2.5**	84.3 $\pm$ 2.1**	81.31 $\pm$ 1.8*

On being exposed to sublethal doses of arsenic, the population of hyalinocytes and granulocytes exhibited a steady decline in 1 ppm of sodium arsenite (Figure 2).

The granulocytes showed a remarkable rise in count against 2 ppm of sodium arsenite up to 30 days (Figure 3). Exposure of the animals in 3 ppm for 4 days resulted a highest increase in distribution of hyalinocytes, 10 times more and an lowest decrease in semigranulocytes, 4 times than control (Figure 4).

Restoration of hyalinocytes, semigranulocytes and granulocytes count of post-treated *S. serrata* maintained in arsenic free water for 30 days (Table 2, 3 and 4). Data showed that maximum recovery of subpopulations of hemocyte was against 1 ppm of sodium arsenite treatment (Table 2). Detailed data are accounted in tables 2, 3 and 4.

Table 2. Restoration of hemocyte hyalinocyte, granulocyte and granulocyte count of post treated (1 ppm of sodium arsenite/4, 15 and 30 days) *S. serrata* maintained in arsenic free water for 30 days. Data expressed as percentage (n =5)

Hemocyte populations subtype	DHC (%) in control	DHC (%) in post-treated <i>S.serrata</i>	DHC (%) of <i>S. serrata</i> maintained in arsenic free water			
		1 ppm/4days	Day 5	Day 10	Day 20	Day 30
Hyalinocyte	7.47	3.07	4.02	5.34	6.09	7.42
Semigranulocyte	65.29	75.17	73.62	69.96	66.14	65.46
Granulocyte	27.24	21.76	23.11	25.16	26.09	27.12
		1ppm/15days				
Hyalinocyte	7.47	2.09	3.97	4.67	5.17	6.94
Semigranulocyte	65.29	79.80	75.08	70.19	67.11	65.32
Granulocyte	27.24	18.11	19.89	22.64	25.67	27.74
		1ppm/ 30days				
Hyalinocyte	7.47	1.17	2.45	4.67	5.41	7.25
Semigranulocyte	65.29	82.62	80.14	72.12	68.06	64.85
Granulocyte	27.24	16.21	18.15	22.68	26.61	27.90

Table 3. Restoration of hemocyte hyalinocyte, granulocyte and granulocyte count of post treated (2 ppm of sodium arsenite/4, 15 and 30 days) *S. serrata* maintained in arsenic free water for 30 days. Data expressed as percentage (n =5)

Hemocyte populations subtype	DHC (%) in control	DHC (%) in post-treated <i>S.serrata</i>	DHC (%) of <i>S. serrata</i> maintained in arsenic free water			
		2 ppm/4days	Day 5	Day 10	Day 20	Day 30
Hyalinocyte	7.47	7.23	7.25	7.35	7.40	7.41
Semigranulocyte	65.29	71.01	70.02	68.86	66.12	65.31
Granulocyte	27.24	21.76	22.64	24.68	26.51	27.28
		2 ppm/15days				
Hyalinocyte	7.47	1.09	2.97	4.07	5.17	6.92
Semigranulocyte	65.29	69.80	68.08	66.86	64.11	65.89
Granulocyte	27.24	28.11	28.09	28.04	27.97	27.19
		2 ppm/30days				
Hyalinocyte	7.47	1.39	2.45	4.67	5.41	6.1
Semigranulocyte	65.29	68.21	67.14	66.12	65.06	63.71
Granulocyte	27.24	30.40	28.45	22.68	26.61	30.19

Table 4. Restoration of hemocyte hyalinocyte, granulocyte and granulocyte count of post treated (3 ppm of sodium arsenite/4, 15 and 30 days) *S. serrata* maintained in arsenic free water for 30 days. Data expressed as percentage (n =5)

Hemocyte populations subtype	DHC (%) in control	DHC (%) in post-treated <i>S.serrata</i>	DHC (%) of <i>S. serrata</i> maintained in arsenic free water			
		3 ppm/4days	Day 5	Day 10	Day 20	Day 30
Hyalinocyte	7.47	69.71	60.95	45.32	20.09	8.21
Semigranulocyte	65.29	15.31	73.62	69.96	66.14	64.81
Granulocyte	27.24	14.48	23.11	24.56	25.59	26.98
		3 ppm/15days				
Hyalinocyte	7.47	11.5	10.17	8.67	5.17	6.94
Semigranulocyte	65.29	63.72	63.98	64.28	64.99	65.32
Granulocyte	27.24	24.78	24.19	25.64	26.67	27.74
		3 ppm/30days				
Hyalinocyte	7.47	9.2	8.55	8.67	8.41	8.25
Semigranulocyte	65.29	63.1	63.81	64.12	64.46	64.85
Granulocyte	27.24	27.7	27.69	27.65	27.62	27.64

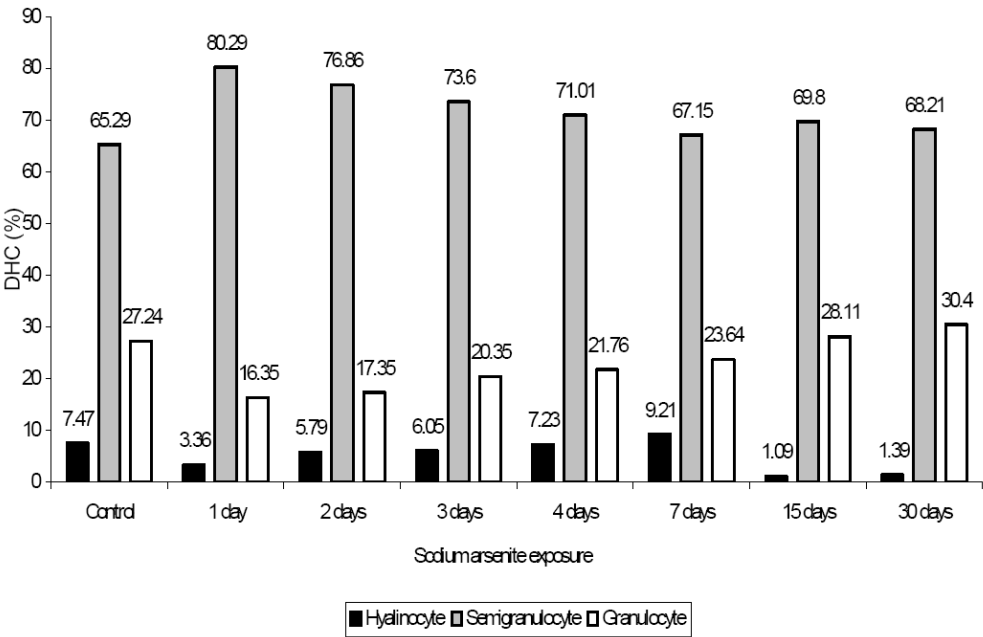


Figure 4. Alteration of differential hemocyte count (DHC) of *S. serrata* under exposure of 3 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15, 30 days. Data expressed as percentage (n =5).

DISCUSSION

Hemocytes not only play an important role in immunological responses but also involve in different physiological functions. The values of THC and DHC considered useful source for other cell biological studies due to enhance the immune capability during periods of stress

leading to disease resistance (Truscott and White, 1990; Saha and Ray, 2006). According to Aggett and Kriegman (1988) during summer the pentavalent, arsenate is chemically reduced to 60 times more toxic trivalent, arsenite in the mud-water interface and according to Das and Roy Choudhury (2006) natural aquatic environmental concentration of arsenic in West Bengal is 3.2 ppm. So natural habit of mud crab, *S. serrata* is directly contaminated by metalloid arsenic. As a result continuous fitting in biounsafe environment are under constant threat of physiological stress by different species of xenobiotics including arsenic. Exposure of *S. serrata* to 1, 2 and 3 ppm of sodium arsenic for 1, 2, 3, 4, 7, 15 and 30 resulted an alteration of enumeration of hemocyte density and subpopulations which is directly proven the stress conditions of the specimens nearly same as natural habitat. Increment of THCs indicates more proliferation of hemocytes that demonstrated the activation of hemocytopoietic organs of crab to release more hemocytes in blood stream (Victor, 1993). This dramatic situation may lead to maintain the stress condition of *S. serrata*. Partial recovery of THC and DHC of hemocytes of post-treated crabs indicated the residual toxicity of arsenic which was accumulated in different parts of animal's body. Chronic exposure of arsenic may lead to permanent alteration of immunological status in *S. serrata*. Such situation would result of impairment of immunological activities and dysfunction of hemocyte. *S. serrata* may become susceptible to microbial infection leading to mass mortality and steady decline of this important biological resource from Sundarbans Biosphere Reserve. Present study is related with the public health safety and aimed to provide baseline information in formulating a preliminary suitable strategy of aquaculture of *S. serrata* in the geographic regions contaminated by arsenic for human consumption.

ACKNOWLEDGMENT

Head of the Department of Zoology, University of Calcutta is thankfully acknowledged for providing the departmental instrument and allied facilities funded by DST (FIST) and UGC (SAP).

REFERENCES

- Ali, M. Y., Kamal, D., Hossain, S. M. M., Azam, M. A., Sabbir, W., Murshida, A., Ahmed, B. and Azam, K. (2004). Biological studies of the mud crab, *Scylla serrata* (Forsk.) of the Sundarbans mangrove ecosystem in Khulna region of Bangladesh. *Pakistan J. Biol. Sci.*, 7(11): 1981-1987.
- Aggett, J. and Kriegman, M. R. (1988). The extent of formation of arsenic (III) in sediment interstitial waters and its release to hypolimnetic waters in lake Ohakuri. *Waters Res.*, 22(4): 407.
- Chaudhuri, A. B. and Choudhury, A. (1994). Mangrove of Sundarbans Vol. I: India (Zakir Hussain, M. and Acharya, G. Ed.) IUCN, Bangkok, Thailand. The World Conservation Union.
- Chakraborty, S., Ray, M. and Ray, S. (2008). Sodium arsenite induced alteration of hemocyte density of *Lamellidens marginalis* - an edible mollusk from India. *Clean*, 36 (2): 195-200.

- Das, S. and Roy Chowdhury, A. (2006). Arsenic: a global monster. *Sci. Cult.*, 72(7- 8): 230 – 237.
- Ghosh S., Ray M. and Ray S. (2008). Kinetics of nonself adhesion and viability of immunocytes of blood of economically important Mollusc, *Telescopium telescopium* of Sundarbans estuary. *Icfai J. Life Sci.*, 2 (3):7 – 14.
- Gomez - Caminero, A., Howe, P., Hughes, M., Kenyon, E., Lewis, D. R., Moor, M., Nag. J., Aitio, A. and Becking, G. (2001). Summary. In Environmental Health Criteria 224, Arsenic and Arsenic Compounds (Ed: 2nd), pp. 1-8: World Health Organization, Geneva.
- Heasman, M. P. and Fielder, D. R. (1983). Laboratory spawning and mass rearing of the mangrove crab (*Scylla serrata* Forskal), from first zoea to first crab stage. *Aquacult.*, 34: 303 - 316.
- Klassen, C. D. (1991). Principles of toxicology. In: Gilman, A. G., Tall, T. W., Nies, A. S., Taylor, P. (Eds.), *Pharmacological Basis of Therapeutics*, 8th ed. McGraw Hill, pp. 49 – 61.
- Krishnaja, A. P., Rege, M. S. and Joshi, A. G. (1987). Toxic effects of certain heavy metals (Hg, Cd, As, Se) on the intertidal crab *Scylla serrata*. *Mar. Environ. Res.*, 21(2): 109 – 119.
- Mukherjee, S., Ray, M. and Ray, S. (2008). Dynamics of hemocyte subpopulation of *Lamellidens marginalis* exposed to a neem based pesticide. In *Zoological Research in Human Welfare*, ZSI, Kolkata Pub., paper 40: 389 – 394.
- Pal, T., Mukherjee, P. K., Sengupta, S., Bhattacharyya, A. K. and Shome, S. (2002). Arsenic pollution in groundwater of West Bengal, India – an insight into the problem by subsurface sediment analysis. *Groundwater research*, 5(2): 501 – 512.
- Saha, S. and Ray, S. (2006). Hemocyte profile of the estuarine mud crab, *Scylla serrata*. *Environ. Ecol.*, 24S (3A): 818 – 819.
- Saha, S., Ray, M. and Ray, S. (2007). Analyses of total count of hemocytes of estuarine crab, under acute arsenic exposure. *Icfai J. Life Sci.*, 1(3): 75 – 78.
- Saha, S., Ray, M. and Ray, S. (2008). Kinetics of nonself surface adhesion and phagocytic response of hemocytes of *Scylla serrata* exposed to sodium arsenite. *Toxicol. Int.*, 15(1): 15 – 19.
- Saha, S., Ray, M. and Ray, S. (2008). Nitric oxide generation by immunocytes of mud crab exposed to sodium arsenite. In *Zoological Research in Human Welfare*, ZSI, Kolkata Pub., paper 44: 425 - 428.
- Sarkar, S. K., Franciskovic – Bilinski, S., Bhattacharya, M., Saha, M. and Bilinski, H. (2004). Level of elements in the surficial estuarine sediments of the Hugli River, northeast India and their environmental implications. *Environ. Int.*, 30 (8): 1089 – 1098.
- Shibu Vardhannan, Y. and Radhakrishnan, T. (2002). Acute toxicity of copper, arsenic and HCH to paddy field crab, *Paratelphusa hydrodromus* (Herb.). *J. Environ. Biol.*, 23(4): 387 – 392.
- Truscott, R. and White, K. N. (1990). The influence of metal and temperature stress on the immune system of crabs. *Funct. Ecol.*, 4: 455 –461.
- Yildiz, H. Y. and Atar, H. H. (2002). Hemocyte classification and differential counts in freshwater crab (*Potamon fluviatilis*). *Turk. J. Vet. Anim.*, 26: 403-406.

IMMUNOTOXICITY OF AZADIRACHTIN IN FRESHWATER MUSSEL IN RELATION TO SURFACE ADHESION OF HEMOCYTES AND PHAGOCYTOSIS

Suman Mukherjee, Mitali Ray and Sajal Ray*

Department of Zoology,
Aquatic Toxicology Laboratory,
University of Calcutta.
35 Ballygunge Circular Road,
Kolkata – 700019, West Bengal ,India.

ABSTRACT

Lamellidens marginalis (Mollusca: Eulamellibranchiata) is a freshwater edible mussel distributed in the wetland of different districts of West Bengal of India. Hemocytes – the blood cells of this species perform diverse immunological functions including nonself recognition and phagocytosis of nonself particles. Assay of nonself surface (glass) adhesion of hemocytes was determined under the exposure of 0.006, 0.03, 0.06 and 0.09 ppm of a biopesticide azadirachtin in diverse span of exposure i.e. 1, 2, 3, 4 and 7 days in controlled laboratory condition. Shifts in percentage of adhesion were recorded against all the concentration screened. However, the patterns of shift were not uniform.

Azadirachtin induced shift in surface adhesion of hemocytes indicated a state of immunological toxicity and stress in the species. Phagocytosis of nonself particulates is considered as a classical immunological response in bivalve. We have examined phagocytosis of yeast (*Saccharomyces cerevisiae*) by hemocytes of mussel exposed to 0.006, 0.03, 0.06 and 0.09 ppm of azadirachtin for 1, 2, 3, 4 and 7 days *in vitro*.

Alteration in phagocytic response and adhesion of hemocytes is suggestive of cellular stress which may lead to decline in population of *L. maginalis* in xenobiotic affected districts of West Bengal of India.

Keywords: Azadirachtin, *Lamellidens marginalis*, hemocytes, biopesticide.

* Corresponding author: E. mail: raysnailmail@rediffmail.com

INTRODUCTION

Lamellidens marginalis, an economically important edible bivalve is distributed in the freshwater ecosystem of West Bengal of India. Hemocytes are the circulating blood cells of bivalve that perform various immunological functions and discriminate the nonself surface through adhesion (Armstrong, 1985; Lackie, 1988; Johansson and Soderhall, 1992; Johansson *et al.*, 1995; Saha and Ray, 2006). Azadirachtin, a new generation biopesticide is recently being introduced in the agricultural sector of India. Azadirachtin is a limonoid compound obtained from a neem plant *Azadirachta indica*. During monsoon season, the agricultural runoff containing azadirachtin residues contaminate the fresh habitat of mussel. In this present study, the immunotoxicity of azadirachtin is reported in *L. marginalis* in relation to phagocytosis and surface adhesion. Nonself surface adhesion of hemocyte was estimated under the exposure of azadirachtin in controlled laboratory condition. A discrete population of hemocyte of invertebrates is capable of recognition and adhesion to various nonself surfaces and considered as immune response (Armstrong, 1980; Johansson and Soderhall, 1989a; Hose *et al.*, 1990; Martin *et al.* 1999). Aquatic invertebrates distributed in polluted environment engulf invading microorganisms and parasites (Shapiro *et al.*, 1977; Bayne, 1990; Factor and Beekman, 1990; Hose *et al.*, 1990; Tyson, 1995; Martin *et al.*, 1999) as innate immune response. Phagocytic responses of hemocytes *in vitro* were screened under the sublethal exposure of azadirachtin in bivalve (Fontaine and Lightner, 1974; Paterson and Steward, 1974; Armstrong and Levin, 1979; Ehlers *et al.*, 1992).

MATERIALS AND METHODS

Adult *L. marginalis* of 9 ± 0.5 cm of length weighing 70 ± 10 gm was collected from selected habitat of district of south 24 Parganas of West Bengal, India and transported to the laboratory. Animals were maintained in standard glass aquaria in batches and fed with chopped *Hydrilla*. The water of the static water environment was replenished in every 24 hours and animals were acclimatized for one week (Heasman and Fielder, 1983). LC_{50} of azadirachtin was determined in *L. marginalis* as 1.8 ppm / 96 hours in static water environment (Krishnaja *et al.*, 1987; Shibu Vardhanan and Radhakrishnan, 2002). The temperature of water kept constant at $28 \pm 2^{\circ}\text{C}$. *L. marginalis* was exposed to 0.006, 0.03, 0.06 and 0.09ppm of azadirachtin in batches along with the control for a span of 1, 2, 3, 4 and 7 days duration. During treatment animal received uniform ration of daylight and checked for mortality and morbidity. Blood was collected aseptically from posterior adductor at a volume not exceeding 1ml per bleed per day. Entire bleeding and collection procedure was carried out at 4°C to prevent cell aggregation. Cell viability was estimated with 2% trypan blue following the principle of dye exclusion. Experiments were carried out with cell suspension with greater than 95 % viable cell. For study of surface adhesion, fixed number of control and treated hemocytes of *L. marginalis* were placed over grease free sterile glass surface and was incubated for a period upto 150 mins at 37°C in humid chamber. Percentage of adherent hemocytes were microscopically determined after a period of cell incubation i.e. 150 mins (Guria *et al.*, 2002). After 150 mins glass surfaces were subjected to gentle jetting of sterile snail saline for collection of nonadherent hemocyte. Ratio of adherent to nonadherent

hemocyte were determined by Neubaur hemocytometer (Armstrong, 1980). For assay of phagocytosis, fixed numbers of viable hemocytes were challenged with freshly cultured yeast at an optimal phagocytic ratio of 1:10. Both cell types were maintained in short term culture system for 6 hours to complete the phagocytosis *in vitro*. Cells were subsequently processed fixed and stained for microscopic observation. Phagocytic index (PI) was determined microscopically (Ehlers *et al.*, 1992; Adamowicz and Wojtaszek, 2001).

$$PI = \frac{\text{Total number of phagocytosed cells}}{\text{Total number of cells}} \times 100 \times \frac{\text{Total number of yeast cells engulfed}}{\text{Total number of phagocytosed Cells}}$$

$$PI = \% \text{ of phagocytosis} \times \text{Average number of yeast cells engulfed}$$

RESULTS

In the controlled population, 91.2 ± 0.3 % of hemocyte adhered over glass surface after incubation for 150 minutes. Hemocytes collected from *L. marginalis* exposed to 0.09 ppm of azadirachtin for 7 days expressed lowest percentage of adherent cell as $50 \pm 0.7\%$ (Figure 2). Hemocytes collected from *L. marginalis* exposed to 0.006 ppm of azadirachtin for 1,2,3,4 and 7 days expressed intermediate values of adherent cell percentage. Animal exposed to 0.03 ppm of azadirachtin for 1,2,3,4 and 7 days of exposure resulted depletion in percentage of adherent hemocyte and highest inhibition was recorded against 0.09 ppm for 7 days exposure (Figure 2). A sharp decrease of cell adherence was recorded against 0.06 ppm of exposure for 1,2,3,4 and 7 days as 81.30 ± 0.2 , 72.54 ± 1.2 , 62 ± 0.8 , 54 ± 0.3 and 49 ± 0.2 % at 150 mins interval of incubation. Exposure of the animal in 0.09 ppm for 1,2,3,4 and 7 days resulted a decrease in distribution of adherent hemocytes at each interval of incubation. At 0.09 ppm of exposure, a steady decrease of adherent cells were recorded against 1,2,3,4 and 7 days of exposure respectively. Pattern of cell adherence were identical in all the experiment. A steady decrease in the percentage of adherent hemocytes were recorded against time scale for all the concentration used. A steady depletion of efficacy of hemocytes for surface adhesion against all the concentrations of biopesticide indicated an alteration in immunoefficacy of hemocyte under pesticide exposure. Impairment of phagocytic response were recorded against all concentration of azadirachtin tested. Highest and lowest inhibition of phagocytic response were determined as $PI\ 30 \pm 2.1$ against 0.09ppm / 7days and 335 ± 1.9 against 0.006 ppm / 1day respectively against the control value of 350 ± 2.5 (Figure 1). Both experiments were repeated for 5 times and the data were interpreted by mean $\pm$ standard deviation and compared in paired t-test at $P < 0.05$ (Bailey, 1959). In our present observation, sublethal concentration of 0.006, 0.03, 0.06 and 0.09 ppm of azadirachtin resulted disruption of recognition of nonself surface by hemocytes of *L. marginalis*. Phagocytic response expressed a decreasing trend (Figure 1) under azadirachtin exposure as evident from PI determined in control and treated sets.

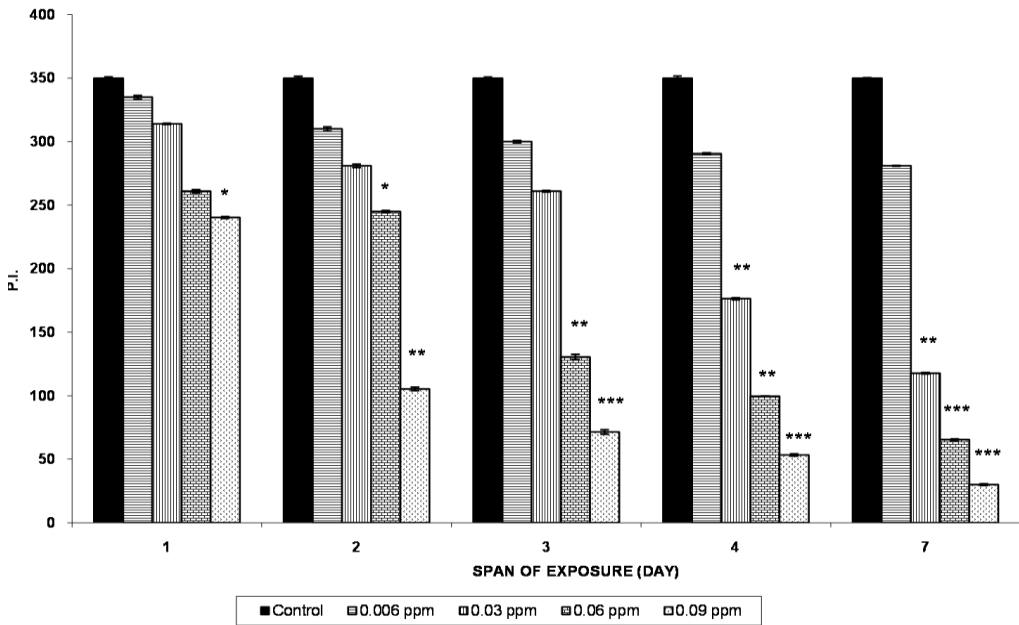


Figure 1. Phagocytic response of hemocytes of azadirachtin exposed *L. marginalis* challenged with yeast. Data is represented as Mean $\pm$ S.D. Statistical significance is shown at $P < 0.05^*$, $P < 0.01^{**}$, $P < 0.001^{***}$.

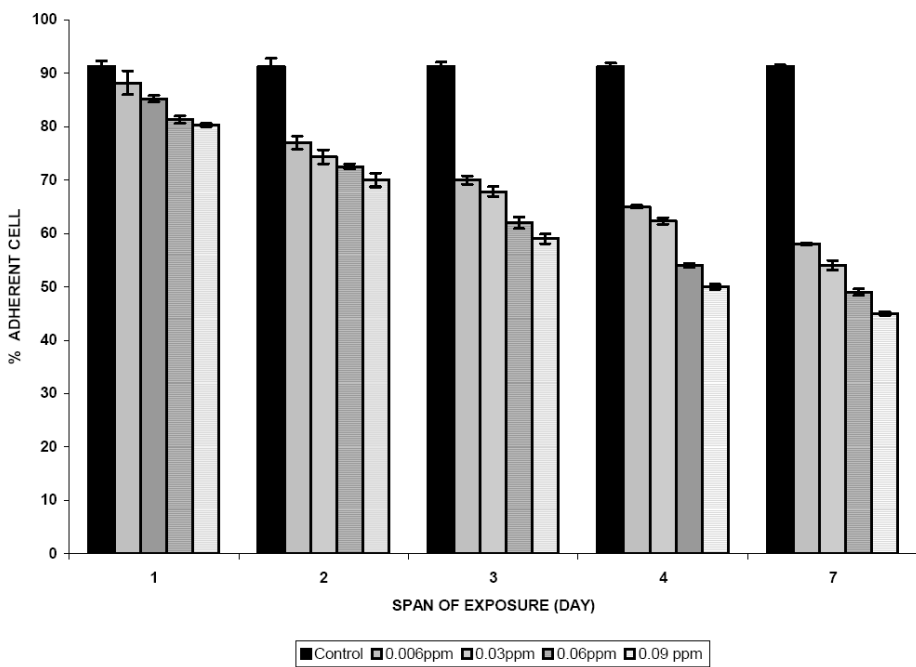


Figure 2. Non self surface adhesion of hemocytes of *L. marginalis* exposed to azadirachtin in vivo. Data is represented as Mean $\pm$ S.D.

DISCUSSION

Hemocytes – the chief effector cells of immunological response of *L. marginalis* are composed of subpopulations namely adherent and nonadherent types (Guria and Ray, 2002). Efficacy of hemocytes for discrimination and adherence over nonself surface was modulated by sublethal concentrations of azadirachtin. Hemocytes of invertebrates phagocytose invading microorganism and pathogen under proper elicitation (Goldenberg *et al.*, 1984; Bayne, 1990). Azadirachtin induced impairment of phagocytic response (Figure 1) indicated a physiological state of immune suppression. Freshwater of West Bengal receives diverse xenobiotics. Alteration of nonself surface adhesion and phagocytic response of hemocyte of *L. marginalis* is indicative of possible immunological impairment which may lead to decline of this economically important species in its natural habitat.

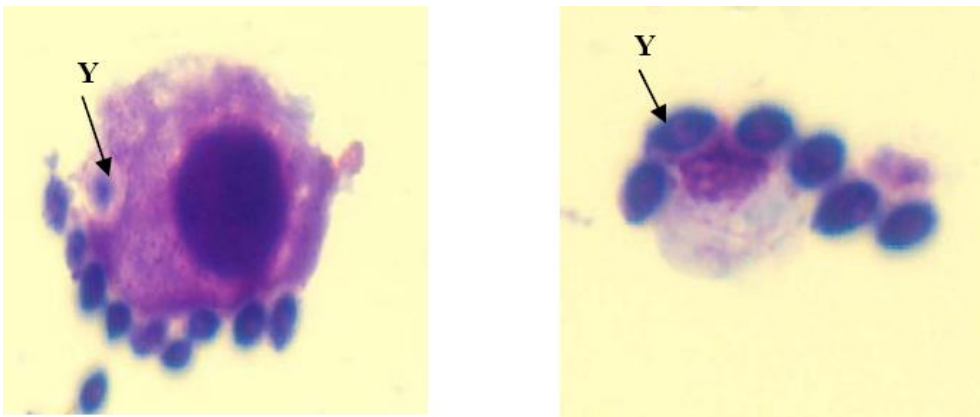


Figure 3. Phagocytosis of yeast (Y) by hemocytes of *L. marginalis*. Control.

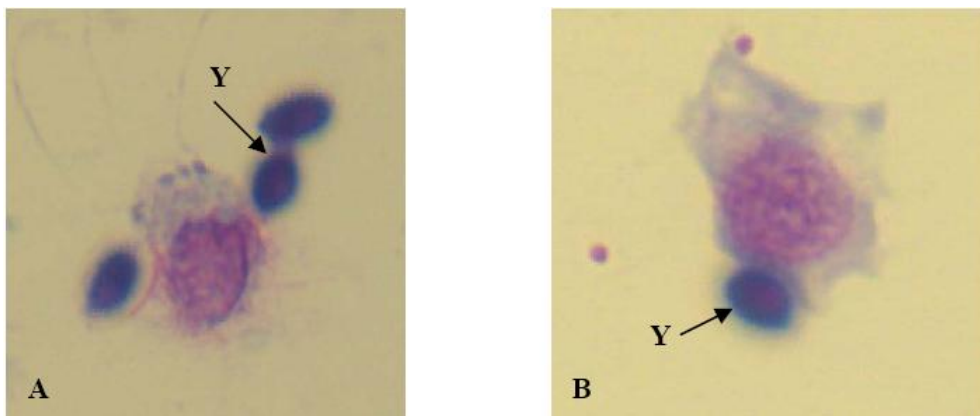


Figure 4. Phagocytosis of yeast (Y) by azadirachtin exposed hemocytes of *L. marginalis*. [(A). 0.006ppm / 7 days, (B). 0.09ppm / 7 days].

ACKNOWLEDGMENT

The authors sincerely acknowledge the financial support received from DST, FIST and UGC, SAP of Government of India.

REFERENCES

- Adamowicz, A and Wojtaszek, J (2001): Morphology and phagocytotic activity of coelomocytes in *Dendrobaena veneta* (Lumbricidae). *Zoologica Poloniae* 46(1 – 4), 91 – 104.
- Armstrong, PB and Levin, J (1979): *In vitro* phagocytosis by *Limulus* blood cells. *J. Invert. Pathol.* 34, 145 – 151.
- Armstrong, PB (1980): Adhesion and spreading of *Limulus* blood cells to artificial surface. *J. Cell Sci.* 44, 243 – 262.
- Armstrong, PB (1985): Adhesion and motility of the blood cells of *Limulus*. In *blood cells of marine invertebrates: Experimental systems in cell Biology Comparative Physiology*. W. D. Cohen, editor. Alan R. Liss Inc., New York. 77- 124.
- Bayne, CJ (1990): Phagocytosis and non-self recognition in invertebrates. *Comp. Immunol.* 40(10), 723 – 731.
- Ehlers, D, Zosel, B, Mohrig, W, Kauschke, E and Ehlers, M (1992): Comparison of *in vivo* and *in vitro* phagocytosis in *Galleria mellonella* L. *Parasitol. Res.* 78, 354 – 359.
- Factor, JR and Beekman, J (1990): The digestive system of the lobster, *Homarus americanus*, III. Removal of foreign particles from the blood by fixed phagocytes of the digestive gland. *J. Morphol.* 206, 293 – 302.
- Fontaine, CT and Lightner, DV (1974): Observations on the phagocytosis and elimination of carmine particles injected into the musculature of the white shrimp, *Penaeus setiferus*. *J. Invert. Pathol.* 24, 141 – 148.
- Guria, K and Ray, S (2002) : Modulation of glass surface adhesion characteristics and sub population shift of hemocytes of *Bellamya bengalensis* by synthetic pyrethroid. *Indian J. Environ. Ecoplan.* 6(1), 175-178.
- Heasman, MP and Fielder, DR (1983). Laboratory spawning and mass rearing of the mangrove crab (*Scylla serrata* Forskal), from first zoea to first crab stage. *Aquacult.* 34, 303-316.
- Hose, JE, Marin, GG and Gerard, AS (1990): A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. *Biol. Bull.* 178, 33 – 45.
- Johanson, MW, Lind, MI, Holmblad, T, Thornqvist, PO and Soderhall, K (1995): Peroxinectin, a novel adhesion protein from crayfish blood. *Biochem. Biophys. Res. Commun.* 216, 1079 – 1087.
- Johansson, MW and Soderhall, K (1989a): A cell adhesion factor from crayfish hemocytes has degranulating activity towards crayfish granular cells. *Insect Biochem.* 19, 183 – 190.
- Johansson, MW and Soderhall, K (1992): Cellular defense and Cell adhesion in crustaceans. *Animal Biol.* 1, 97-107.
- Krishnaja, AP, Rege, MS and Joshi, AG (1987): Toxic effects of certain heavy metals (Hg, Cd, As, Se) on the intertidal crab *Scylla serrata*. *Mar. Environ. Res.* 21(2), 109-119.

- Lackie, AM (1988): Hemocyte Behaviour. *Adv. Insect Physiol.* 21, 85 – 178.
- Martin, GG, Lin, JH and Luc, C (1999): Reexamination of hemocytes in brine shrimp (Crustacea, Branchiopoda). *J. Morphol.* 242, 283 – 294.
- Paterson, W and Stewart, J (1974): *In vitro* phagocytosis by hemocytes of American lobster (*Homarus americanus*). *J. Fish Res. Bd. Can.* 31, 1051 – 1056.
- Saha, S and Ray, S (2006): Hemocyte profile of the estuarine mud crab, *Scylla serrata*. *Environ. and Ecol.* 24S (3A), 818 – 819.
- Shapiro, HC, Steenbergen, JF and Fitzgerald, ZA (1977): Hemocytes and phagocytosis in the American lobster, *Homarus americanus*. *Comp. Pathol.* 3, 126 – 134.
- Shibu Vardhannan, Y and Radhakrishnan, T (2002): Acute toxicity of copper, arsenic and HCH to paddy field crab, *Paratelphusa hydrodromus* (Herb.). *J. Environ. Biol.* 23(4), 387 – 392.
- Tyson, GE (1995): Phagocytosis and digestion of spirochaetes by amebocytes of infected brine shrimp. *J. Invert. Pathol.* 26(1), 105 – 111.

AGGREGATION AND CHEMICAL INDUCED INTERFERENCE OF AGGREGATION OF HEMOCYTES OF INDIAN MUDCRAB EXPOSED TO ARSENIC

Sajal Ray*, Mitali Ray and Sanjib Saha

Aquatic Toxicology Laboratory, Department of Zoology,
University of Calcutta, 35 Ballygunge Circular Road,
Kolkata – 700019, West Bengal, India

ABSTRACT

Sundarbans biosphere reserve of India is an ecologically important zone which shelters diverse forms of animals and plants. *Scylla serrata* (Arthropoda: Crustacea: Decapoda) or estuarine mudcrab is an important consumer of this mangrove ecosystem. Blood or hemolymph of *S. serrata* contains hemocytes, capable of performing diverse physiological functions. Hemocytes the circulating blood cells are considered as immunoreactive agents capable of performing phagocytosis, nonself adhesion and aggregation. Arsenic is a major environmental threat in the specific districts of the state of West Bengal of India. Estuarine water of Sundarbans mangrove bears the risk of arsenic contamination due to anthropogenic and geological consequences. Magnitude of hemocyte aggregation was studied in depth under the exposure of 1, 2 and 3 ppm of sodium arsenite for 1,2,3,4,7,15 and 30 days of exposure. Arsenic exposure yields a dose responsive decrease of hemocyte aggregation against a control level of aggregation of 67.77%. Estuarine ecosystem of Sundarbans mangrove is characteristics of severe intra and interspecific struggle. In this dynamic ecosystem, the inhabitants participate in the struggle of niche occupation for survival and existence. Situation often leads to a state of acute predation and fight among animals. As a result, the animals experience physical wounding and loss of body fluid. Aggregation of hemocyte at wound site prevents the loss of blood and entry of microorganism and considered as an immunological response. Magnitude of aggregation was screened under the experimental exposure of sodium citrate, EDTA, heparin, fructose and mannose at different concentrations. Study was aimed to screen the effect of chelating agent, sugars and anticoagulants on aggregation. For all the chemicals screened, a drastic decrease in the occurrence of free cells were reported which is suggestive to role of these agents in the physiological process of hemocyte aggregation. Moreover exposure to arsenic may lead to gradual loss of blood cell homeostasis of mudcrab distributed in its natural habitat. Continuous exposure to

* Corresponding author: E- mail: raysnailmail@rediffmail.com; Telephone – 91(033)24229800; Fax no. – 91(033)24614849.

toxic arsenic may lead to a population decline of mud crab and loss of biodiversity in this region.

Keywords: Arsenic, Mudcrab, Hemocyte, Mangrove.

INTRODUCTION

Sundarbans biosphere reserves of West Bengal, India support a wide diversity of invertebrates including crabs (Chaudhuri and Choudhury, 1994). UNESCO in 1989, declared this important ecological region as 'Biosphere Reserve' and the region is considered as 'World heritage site' by IUCN. Sundarbans mangrove swamp is an unique ecosystem which is occupied by a varied range of flora and fauna. Tidal submergence and periodic inflow of nutrients characterize this ecosystem as highly dynamic in relation to physiological status of the animals. Estuarine mud crab, *S. serrata* is an important inhabitant of this ecosystem (Ali *et al.*, 2004). This animal is a dietary item of food for human and other consumers.

This delta is in threat of water pollution caused by different anthropogenic activities. Sundarbans delta receives toxins of varied chemistry yet to be characterized in depth (Ghosh *et al.*, 2008). Due to contamination of this estuarine ecosystem, inhabitants of Sundarbans are in continuous exposure to different xenobiotics including arsenic (Pal *et al.*, 2002). Arsenic is an important toxic metalloid which is potentially capable of creating physiological disturbances both at cellular and sub-cellular levels (Das and Roy Chowdhury, 2006; Krishnaja *et al.*, 1987) of invertebrates.

Sublethal toxicity of arsenic in mud crab is a poorly studied area of research which demands special attention from toxicologists and ecologists. Hemocytes are cellular component of the blood or hemolymph and these morphologically discrete cells are the chief component of crustacean immune system (Saha and Ray, 2006). Under the exposure of toxins and parasites, hemocytes of crab are capable of eliciting specific immunological response. Phagocytosis of non-self particulates, recognition of non-self surface and cell-cell aggregation are the distinct responses offered by circulating hemocytes (Rustishauser *et al.*, 1988; Chen *et al.*, 1996; Saha *et al.*, 2008).

Cell-cell aggregation is thought to be an important metabolic process of cell which is poorly understood in crustaceans (Kenney *et al.*, 1972). In this present study, degree of cell aggregation was quantitated under the toxic exposure of arsenic. Simultaneously aggregation *in vitro* was experimentally interfered with ethylene diamine tetraacetic acid (EDTA) (0.0078 to 32 mM), sodium citrate (0.0078 to 32 mM), heparin (20 to 5120 mM), fructose (0.0078 to 32 mM), mannose (0.0078 to 32 mM) under the exposure of sublethal concentration of arsenic.

Study was aimed to analyze the degree of aggregation, magnitude of interference under the exposure of 1, 2 and 3 ppm of sodium arsenite. Information will provide an information base in understanding the degree of hemocytes aggregation in presence of sublethal concentration of arsenic.

MATERIALS AND METHODS

Live adult specimens of *S. serrata* weighing 65-75 gm were collected from selected habitats, which are free from natural arsenic contamination. Length and breadth of the carapace of experimental animals were 6 ± 0.5 cm and 9 ± 0.5 cm respectively. Live crabs were transported to the laboratory in rectangular plastic boxes with water (Saha and Ray, 2006). Live animals were maintained in arsenic free estuarine water and fed with fresh flesh of molluscs (*Bellamya bengalensis*) once a day. The water of glass aquaria was replenished in every 24 hours to avoid residual toxicity. Animals were acclimatized in laboratory conditions for 6-8 days prior to experimentation. During experiments, experimental subjects received uniform ration of illumination and temperature of water ranged from 25 - 30 °C (Heasman *et al.*, 1983). Aqueous solution of sodium arsenite (E. Merck, Germany; 99% pure; CAS number 7784-46-5) was prepared with measured volume of arsenic free distilled water. Treatment was carried out in batches of mudcrab maintained in rectangular borosilicate containers. Containers were fitted with electrically operated aerator to facilitate supply of atmospheric oxygen. Morbidity and mortality of mudcrab were routinely checked in control and treatment set for the entire period of experiment. Determination of median lethal concentration (LC₅₀) of sodium arsenite was carried out after Klassen (1991). Fresh hemolymph of *S. serrata* was collected aseptically from the base of one of the second walking legs of crab by sterile needle. Collected blood was stored in prechilled sterile glass vials to avoid cell aggregation and the volume of collected blood of each animal did not exceed 0.5 ml at one extraction (Yildiz and Atar, 2002). Suspended hemocytes were sedimented by cold centrifugation (Hermle, High Speed Refrigerated centrifuge, Germany) of the hemolymph at 3000 rpm for 5 minutes. Cell density was adjusted with uniform volume of sterile phosphate buffered saline (PBS) as and when necessary. Free and live hemocytes in suspension were observed and screened under inverted microscope (Axiovert, Zeiss, Germany). Cell viability was tested by staining the cell with 0.4% trypan blue (Himedia, India) following the principle of dye exclusion. Cell enumeration was carried out by Neubauer hemocytometer (Germany). Total number of viable hemocytes was determined microscopically by estimating the percentage of stained and unstained cells (Suave *et al.*, 2002). Experiments were carried out with cell suspension with greater than 95 % viable hemocytes. Aggregation of hemocytes *in vitro* was studied in crabs exposed to 1, 2 and 3 ppm of sodium arsenite along with control. Fixed volumes of hemolymph with uniform density of hemocytes were placed on two different chilled microfuge tubes for each untreated and treated samples. One of the samples was fixed by 2.5% of glutaraldehyde (SRL, India). To obtain values for “no aggregation,” hemolymph was treated with 2.5% glutaraldehyde to arrest cell aggregation. The total free cell number was estimated in both control and treated hemolymph after Chen and Bayne (1995). Aggregating hemocytes of *S. serrata* were subjected to the exposure of various chemical inhibitors of cell i.e. EDTA (SRL, India), sodium citrate (SRL, India), heparin (SRL, India), fructose (SRL, India) and mannose (SRL, India) (Kenney *et al.*, 1972; Chen and Bayne, 1995).

Resulting interference were estimated by determining the percent inhibition of hemocyte aggregation. Experiments were repeated for 5 times and values are expressed as percentages of cell aggregation which were determined microscopically.

RESULTS

Alteration in hemocytes aggregation was recorded under the exposure of 1, 2 and 3 ppm of sodium arsenite for 1, 2, 3, 4, 7, 15 and 30 days against control (Figure 1 and 2).

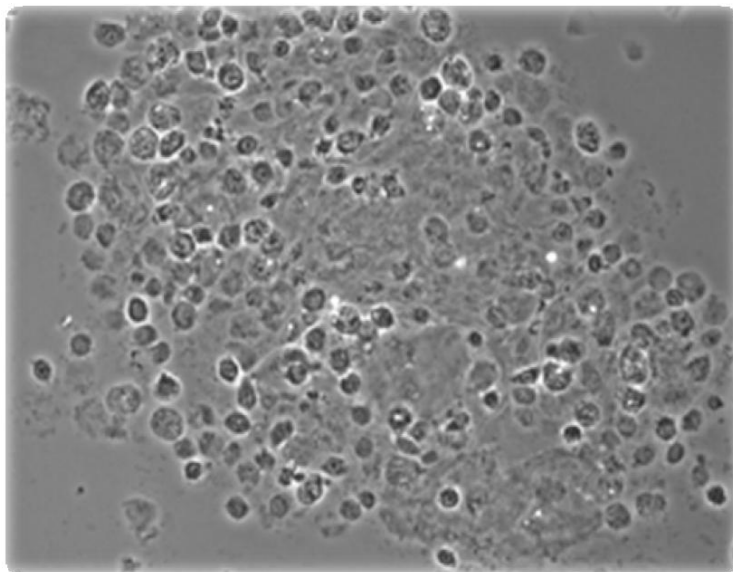


Figure 1. Aggregating hemocytes of *S. serrata*.

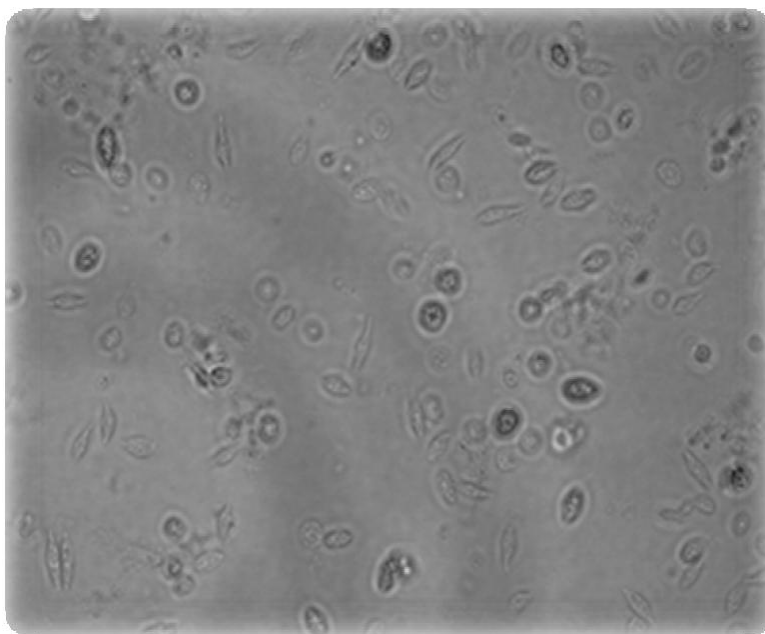


Figure 2. Hemocytes maintained in short term culture.

Kinetics of aggregation indicated a steady increase of free cells under the exposure of arsenic and the lowest value of aggregation of hemocytes was recorded as 9.32 % against the control value of 67.77 % (Figure 3). Hemocytes collected from *S. serrata* exposed to 3 ppm of arsenite expressed lowest percentage of aggregating cells in 1, 2, 3, 4, 7, 15 and 30 days of exposure as 48.51%, 43.02%, 39.43%, 34.43%, 30.45%, 21.33% and 9.32% in respect to 1 and 2 ppm of sodium arsenite. Hemocytes collected from *S. serrata* exposed to 2 ppm of arsenite expressed an intermediate response of aggregation in respect to 1 and 3 ppm of arsenite. Animal exposed to 1ppm of arsenite for 1 day resulted lowest percentage of free hemocyte and lowest inhibition of aggregation.

Interference of hemocytes aggregation was studied extensively by treating the cells with chelating agent (EDTA), anticoagulants (sodium citrate, heparin) and carbohydrates (fructose, mannose) with varying concentrations to determine highest and lowest values of free cells in respect to control (Figures 4, 5, 6, 7 and 8). All the agents resulted a shift in interference in comparison to that of control. Arsenic induced modulation of interference of aggregation was recorded under the exposure of 1, 2 and 3 ppm of sodium arsenite for 30 days (Figures 4, 5, 6, 7 and 8). Sodium arsenite increased the production of free cells in all concentrations of chemicals. A concentration of 32 mM was found to be the effective concentration of 100% inhibition of hemocytes aggregation against EDTA, sodium citrate, fructose and mannose. Whereas for heparin, 5120 unit was the effective concentration for production of 99% free cells (Figure 6).

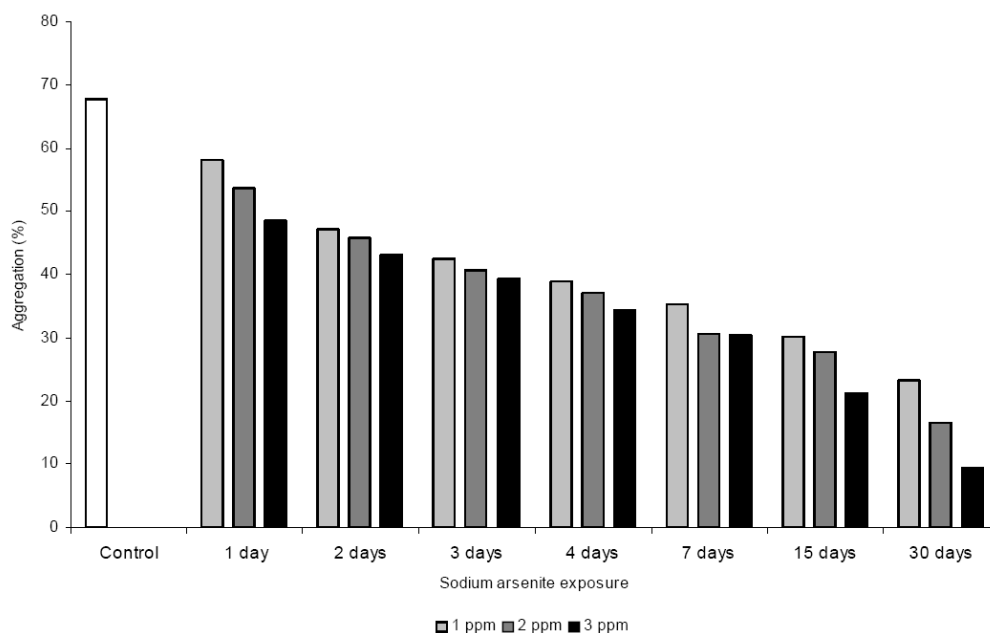


Figure 3. Modulation of aggregation of hemocyte of *S. serrata* under exposure of 1, 2 and 3 ppm of sodium arsenite *in vivo* for 1, 2, 3, 4, 7, 15, 30 days. Data expressed as mean percentage (n =5).

Data is indicative to arsenic induced modification of hemocyte surface characters. Cell surface sugar residues of immunoactive cells play an important role in epitope expression and functioning. Many of these cells express surface receptors for carbohydrates. Study of aggregation interference was carried out in presence of fructose and mannose. Both fructose

and mannose resulted interference of aggregation of hemocytes of *S. serrata* under the exposures of 1, 2 and 3 ppm for 30 days. Treatment of hemocytes with chelating agent like EDTA expressed the same trend of result.

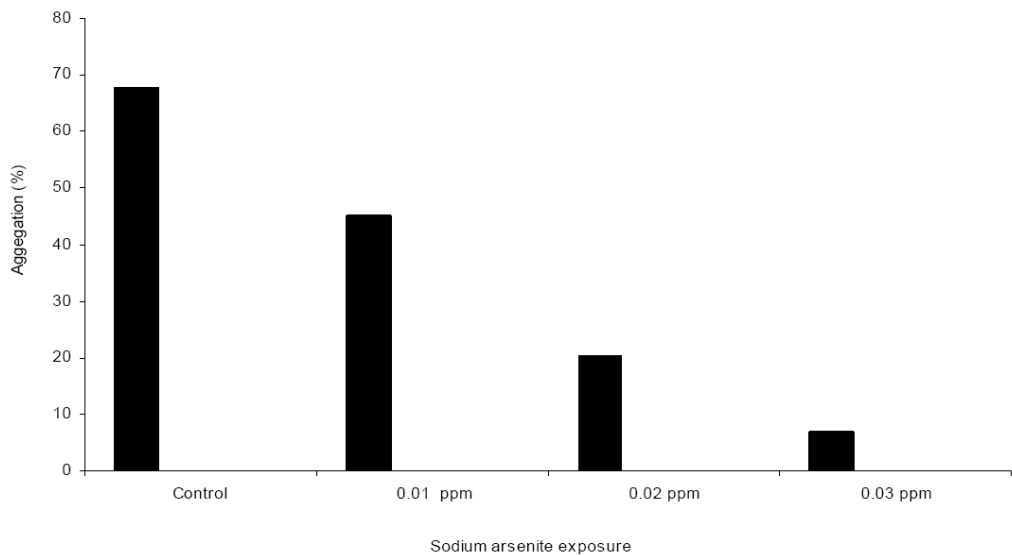


Figure 4. Modulation of aggregation of hemocyte of *S. serrata* under exposure of 0.01, 0.02 and 0.03 ppm of sodium arsenite *in vitro*. (n = 5).

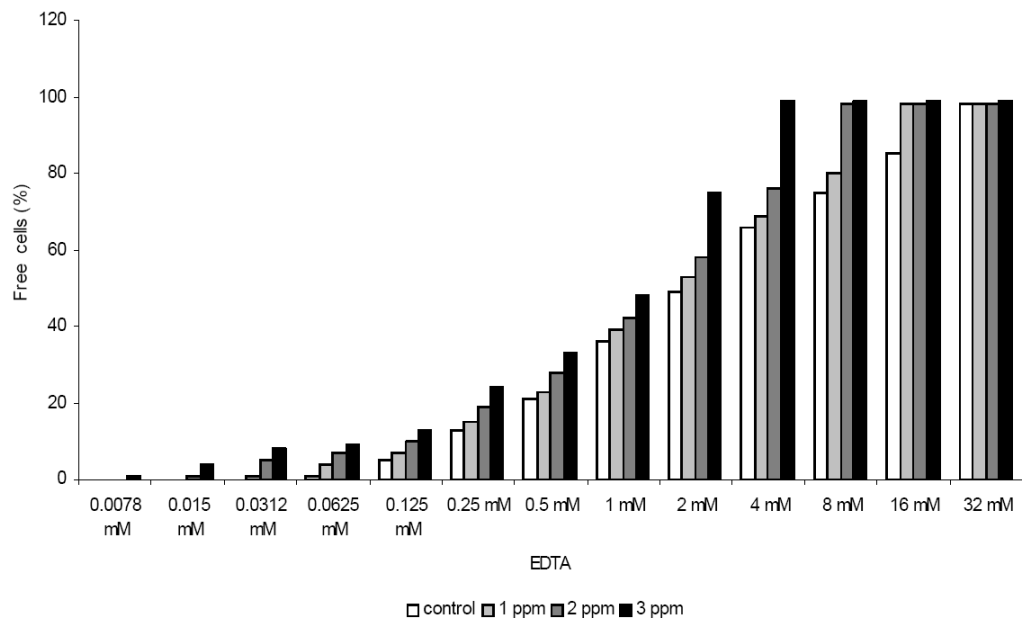


Figure 5. EDTA induced interference of hemocyte aggregation of *S. serrata* exposed to 1, 2 and 3 ppm of sodium arsenite for 30 days (n=5).

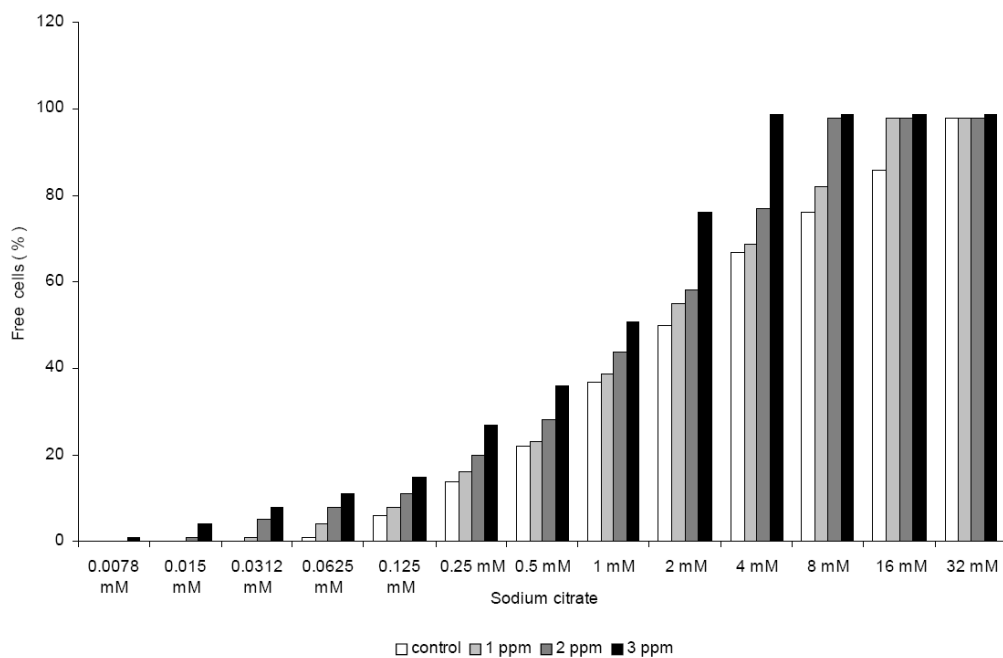


Figure 6. Sodium citrate induced interference of hemocyte aggregation of *S. serrata* exposed to 1, 2 and 3 ppm of sodium arsenite for 30 days (n=5).

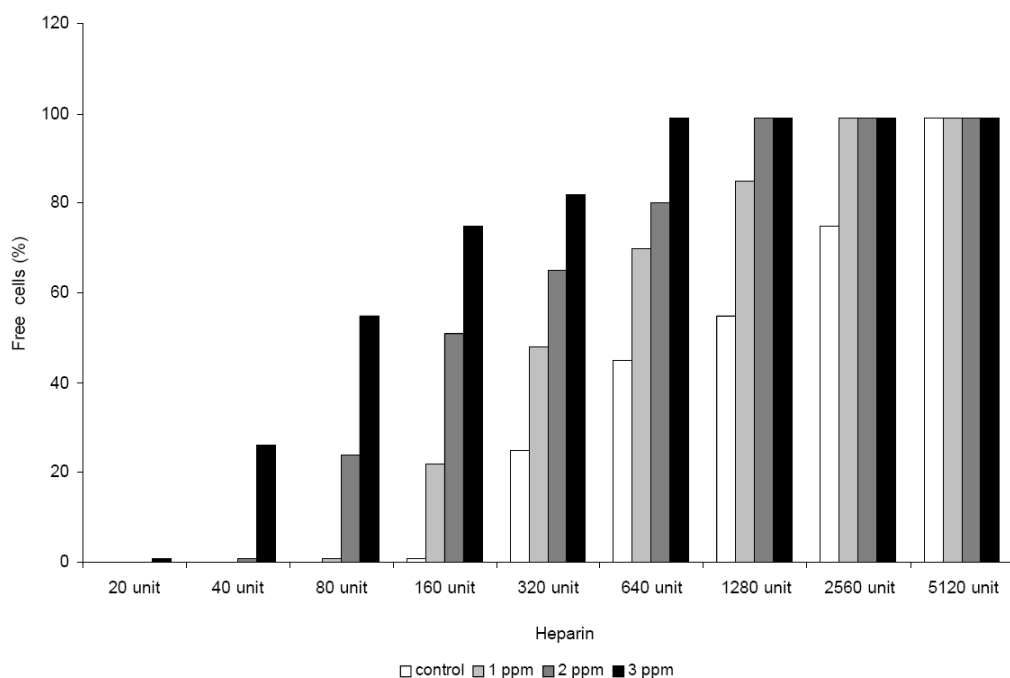


Figure 7. Heparin induced interference of hemocyte aggregation of *S. serrata* exposed to 1, 2 and 3 ppm of sodium arsenite for 30 days (n=5).

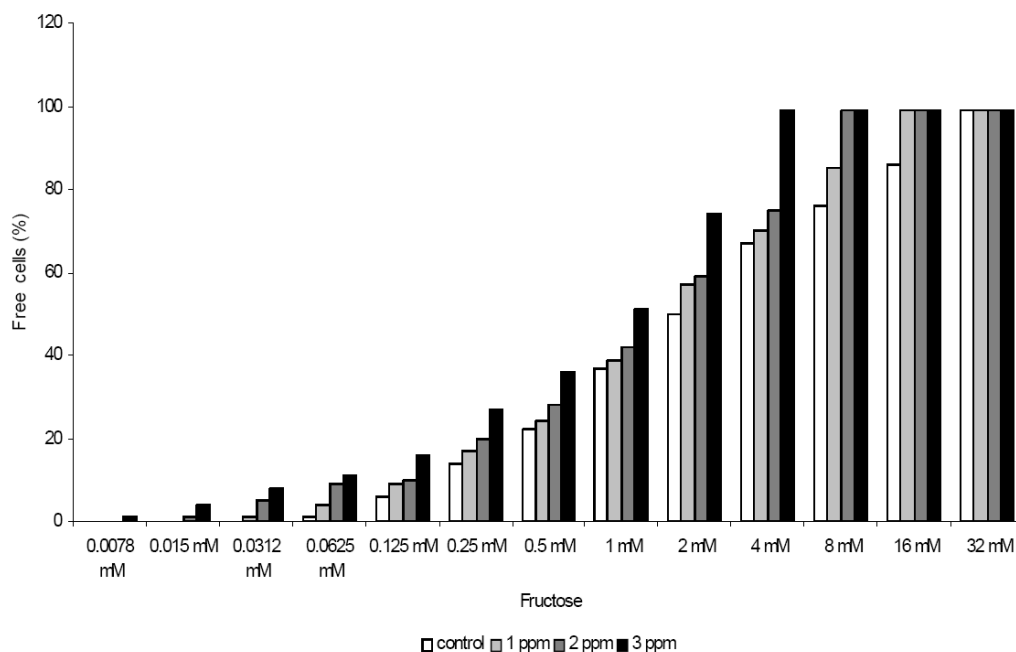


Figure 8. Fructose induced interference of hemocyte aggregation of *S. serrata* exposed to 1, 2 and 3 ppm of sodium arsenite for 30 days (n=5).

DISCUSSION

S. serrata is an economically and ecologically important species of Sundarbans Biosphere Reserve. This species is a significant consumer of estuarine ecosystem and a source of human diet. Mudcrab experiences subchronic and chronic exposures of arsenic residues in its natural habitat. In this present experiment, hemocyte aggregation was screened in depth under the exposure of arsenic and interference of aggregation was studied under the treatment of EDTA, sodium citrate, heparin, fructose and mannose. Aggregation of hemocytes of invertebrates is considered as an important metabolic function and is not examined in *S. serrata* earlier. In normal physiological condition, 67.77% of hemocytes expressed cell aggregation (Figure 4) *in vitro*. Exposure to arsenic expressed a steady decrease in aggregation which showed a dose dependent response. Exposure to 0.03 ppm of arsenic yielded a lowest of cell aggregation as 6.95%. Arsenic induced inhibition of aggregation is indicative to cellular stress and breakdown of blood homeostasis. Arsenic treated hemocytes, when exposed to varying concentrations of EDTA, sodium citrate, heparin, fructose and mannose resulted dose responsive aggregation (Figures 6, 7, 8 and 9). A lowest concentration of 0.0078 mM of EDTA, sodium citrate, fructose and mannose yielded highest degree of cell aggregation, whereas the lowest concentration of 20 unit of heparin expressed the highest aggregation response (Figure 7). Optimum aggregation of hemocyte in the hemolymph of *S. serrata* is a normal physiological phenomenon. Cell-cell attachment is a significant cell behaviour involved in the process of intracellular communication. Most of cellular aggregation is mediated through divalent cations or sugar residues as evident from studies carried out in other invertebrate species (Takahashi *et al.*, 1994).

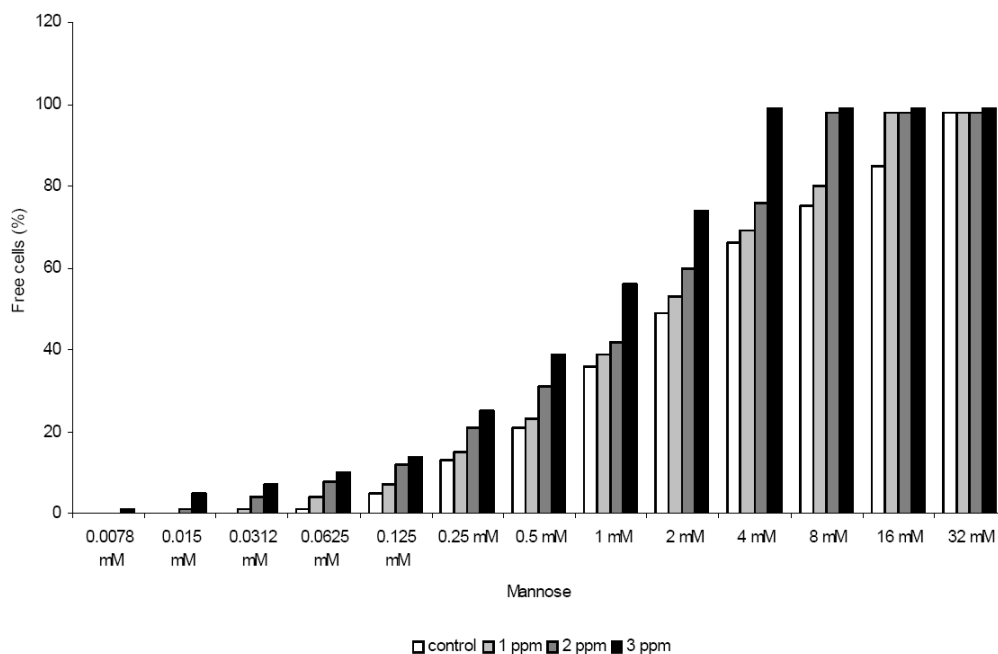


Figure 9. Mannose induced interference of hemocyte aggregation of *S. serrata* exposed to 1, 2 and 3 ppm of sodium arsenite for 30 days (n=5).

In this experiment arsenic treated hemocytes were exposed to specific chemical agents for screening of the shift in aggregation magnitude. Stepwise decrease of occurrence of free cell under the exposure of studied chemicals is indicative of possible role of divalent cations and sugars in the physiological event of intercellular attachment. Interestingly, for all compounds screened, interference of aggregation was highest against lowest concentration. Arsenic, being an important xenobiotics of Sundarbans estuary affect the hemocytes in respect to their metabolic status in terms of aggregation. The control level of occurrence of free hemocytes was less in comparison to experimental exposure to EDTA (Figure 5), sodium citrate (Figure 6), heparin (Figure 7), fructose (Figure 8) and mannose (Figure 9). Increment of aggregation response by all 5 interfering agents and arsenic were indicative to a shift in the surface characteristics of hemocytes of mudcrab. Exposure of mudcrab to arsenic *in vivo* (Figure 3) and *in vitro* (Figure 4) resulted a chronic stress at the level of hemocyte function. Both *in vivo* and *in vitro* exposure resulted a gross decrease in aggregation response of hemocytes. In the competitive environment of estuary, animals struggle for food, space and mate and are often subjected to accidental tissue injury. Hemocyte aggregation is reported to be a major mechanism of clot formation at the site of injury arresting the loss of blood (Holmblad and Soderhall, 1999; Theopold *et al.*, 2004). Arsenic induced inhibition of hemocyte aggregation is a precarious physiological condition which may lead to death of the species following tissue injury. Sundarbans estuary, being susceptible of arsenic pollution appears to be an unsafe habitat of mudcrab unless the level of environmental contamination is controlled. EDTA, Sodium citrate, heparin, fructose and mannose interfered with hemocyte aggregation at different concentrations. Data is suggestive of positive roles of ions, sugars and anticoagulants in the cellular process of hemocyte aggregation of mudcrab. Physiological maintenance of homeostatic concentrations of mono and divalent ions, fructose, mannose and

EDTA in the body fluids of mudcrab is essential for its survival in the estuarine environment of Sundarbans Biosphere Reserve.

ACKNOWLEDGMENT

Author thankfully acknowledge Department of Science and Technology and University Grants Commission of Government of India for providing financial support through FIST and SAP - DRS respectively for instrumentation and other facilities. The ethics committee and environmental biology and toxicology laboratory of the Department of Zoology of University of Calcutta are also gratefully acknowledged.

REFERENCES

- Ali, M. Y., Kamal, D., Hossain, S. M. M., Azam, M. A., Sabbir, W., Murshida, A., Ahmed, B. and Azam, K. (2004). Biological studies of the mud crab, *Scylla serrata* (Forsk.) of the Sundarbans mangrove ecosystem in Khulna region of Bangladesh. *Pakistan J. Biol. Sci.*, 7 (11): 1981-1987.
- Chaudhuri, A. B. and Choudhury, A. (1994). Mangrove of Sundarbans Vol. I: India (Zakir Hussain M and Acharya G Ed.) IUCN, Bangkok, Thailand. The World Conservation Union.
- Chen, J. H. and Bayne, C. J. (1995). Bivalve (molluscs) hemocyte behaviours: characterization of hemocyte aggregation and adhesion and their inhibition in the California mussel (*Mytilus californianus*). *Biol. Bull.*, 188: 255 – 266.
- Chen, J. H., Yang, H. Y. Y., Peng, S. W., Chen, Y. J., Tsai, K. Y. (1996). Characterization of abalone (*Haliotis diversicolor*) hemocytes *in vitro*. *Biol. Bull. NTNU.*, 31(1): 31 – 38.
- Das, S. and Roy Chowdhury, A. (2006). Arsenic: a global monster. *Sci. Cult.*, 72(7- 8): 230 – 237.
- Ghosh, S., Ray, M. and Ray, S. (2008). Kinetics of nonself adhesion and viability of immunocytes of blood of economically important Mollusc, *Telescopium telescopium* of Sundarbans estuary. *Icfai J. Life Sci.*, 2 (3): 7 – 14.
- Heasman, M. P. and Fielder, D. R. (1983). Laboratory spawning and mass rearing of the mangrove crab (*Scylla serrata*, Forskal), from first zoea to first crab stage. *Aquacult.*, 34: 303-316.
- Holmblad, T. and Soderhall, K. (1999). Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquacult.*, 172: 111 – 123.
- Kenney, D. M., Belamarich, F. A. and Shepro, D. (1972). Aggregation of horseshoe crab (*Limulus polyphemus*) amebocytes and reversible inhibition of aggregation by EDTA. *Biol. Bull.*, 143: 548 – 567.
- Klassen, C. D. (1991). Principles of toxicology. In: Gilman A G, Tall T W, Nies A S, Taylor P (Eds.), *Pharmacological Basis of Therapeutics*, 8th ed. McGraw Hill, pp. 49 – 61.
- Krishnaja, A. P., Rege, M. S. and Joshi, A. G. (1987). Toxic effects of certain heavy metals (Hg, Cd, As, Se) on the intertidal crab *Scylla serrata*. *Mar. Environ. Res.*, 21(2): 109 – 119.

- Pal, T., Mukherjee, P. K., Sengupta, S., Bhattacharyya, A. K. and Shome, S. (2002). Arsenic pollution in groundwater of West Bengal, India – an insight into the problem by subsurface sediment analysis. *Groundwater research*, 5(2): 501 – 512.
- Rustishauser, U., Acheson, A., Hall, A. K., Mann, D. M. Sunshine, J. (1988). The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interaction. *Sci.*, 40: 53 – 57.
- Saha, S. and Ray, S. (2006). Hemocyte profile of the estuarine mud crab, *Scylla serrata*. *Environ. Ecol.*, 24S (3A): 818 – 819.
- Saha, S., Ray, M. and Ray, S. (2008). Kinetics of nonself surface adhesion and phagocytic response of hemocytes of *Scylla serrata* exposed to sodium arsenite. *Toxicol. Int.*, 15(1): 15 – 19.
- Sauve, S., Brousseau, P., Pellerin, J., Morin, Y., Senechal, L., Goudreau, P. and Fournier, M. (2002). Phagocytic activity of marine and freshwater bivalves: *in vitro* exposure of hemocytes to metals (Ag, Cd, Hg and Zn). *Aquat. Toxicol.*, 58: 189 – 200.
- Takahashi, H., Azuma, K., Yokosawa, H. (1994). Hemocyte aggregation in the solitary ascidian *Halocynthia roretzi*: plasma factors, magnesium ion and met-lys-bradykinin induced the aggregation. *Biol. Bull.*, 186: 247 – 253.
- Theopold, U., Schmidt, O., Soderhall, K. and Dushay, S. (2004). Coagulation in arthropods: defence, wound closure and healing. *Trends in Immunol.*, 25(6): 289 – 294.
- Yildiz, H. Y. and Atar, H. H. (2002). Hemocyte classification and differential counts in freshwater crab (*Potamon fluviatilis*). *Turk. J. Vet. Animal*, 26: 403-406.

DEVELOPMENTAL EXPRESSION PROFILE OF COCKROACH VITELLOGENIN GENES Vg 1 AND 2

***Azza Mohamed Elgendy*, Mohamed Elmogy*,
Muhammed Tufail and Makio Takeda***

Graduate School of Science and Technology,
Kobe University, Japan T: 657-8501

ABSTRACT

The fat body of female *Periplaneta americana* is the main site of vitellogenins (Vgs) synthesis. The Vgs are large molecules synthesized in the fat body and encoded by two different Vg genes (Vg1 and Vg2). The present study indicates that both Vg genes are represented in the genome by a single copy as revealed by Southern blot analysis.

The Vg2 gene is predominantly expressed in the fat body cells and its expression preceded that of Vg1 gene. The expression profile and level of Vg genes are found to be synchronized and up-regulated by the hemolymph JH titer and suppressed by the ecdysteroid titer during vitellogenesis, as revealed by RT-PCR and real time PCR analysis.

The differences in the expression levels of Vg genes, suggest the possible differences between the two genes in their responsiveness to the hemolymph JH titer and will pave the way toward further investigation on the JH responsive elements that regulate the expression of these two genes.

Keywords: *Periplaneta americana*; female fat body; vitellogenin genes, copy number; real time-PCR, hormones.

INTRODUCTION

All oviparous species, including insects, provision their eggs with vitellin (Vn) as a major yolk protein. The precursor vitellogenin (Vg) is synthesized extra-ovarially by the fat body in tissue-, sex-, and stage-specific manners, secreted into the hemolymph and then sequestered by competent oocytes via receptor-mediated endocytosis (Raikhel and Dhadialla, 1992; Don-Wheeler and Engelmann, 1997; Sappington and Raikhel, 1998). Vgs are large molecules

*Current address: Cairo University, Faculty of Science, Department of Entomology, Giza, Egypt.

(200 kD) synthesized in the fat body in a process that involves substantial structural of the nascent protein prior to its secretion in the hemolymph and transport to the ovaries. The *Vg* modifications (e.g., glycosylation, lipidation, phosphorylation, and proteolytic cleavage, etc.) molecules are cleaved proteolytically and co- and post-translationally modified to facilitate the transport of carbohydrates, lipids, and other nutrients to the ovaries. After incorporation into oocytes, the *Vgs* are stored in a crystalline form as *Vns*, a reserve food-source for the future embryo. The synthesis of *Vg* is regulated at the transcriptional level (Raikhel et al., 2004). The hormones involved in *Vg* gene regulation include juvenile hormone (JH), ecdysone, and several neuropeptides (Engelmann, 1983; Hagedorn, 1985; Wyatt and Davey, 1996; Engelmann and Mala, 2005).

Insects can be divided into three types based on hormonal regulation of the *Vg* gene transcription. Type I includes insects that use only JH for *Vg* gene expression, such as *Leucophaea maderae* (Engelmann, 2002); *Locusta migratoria* (Edwards et al., 1993); *Blattella germanica* (Belles, 1998, 2005; Comas et al., 2001); *Nauphoecata cinerea* (Imboden et al., 1987) and lubber grass hopper (Borst et al., 2000). Type II includes insects which require both JH and ecdysone for *Vg* gene expression, such as *Aedes aegypti*, *Anopheles strephensi*, and *Drosophila melanogaster* (Hagedorn et al., 1985, Raikhel et al., 1992, Soller et al., 1999). Type III includes some lepidopterans that require JH, ecdysteroids, and additional hormones to regulate their reproductive physiology, such as *Choristoneura fumifera* (Delisle and Cusson, 1999) and *Manduca sexta* (Ishizaka et al., 1989).

In hemimetabolous insects such as *Locusta migratoria* (Irvine and Brasch, 1981; Nair et al., 1981; Zhang et al., 1996) and *Leucophaea maderae* (della-Cippa and Engelmann, 1984) JH secreted by corpora allata, stimulates vitellogenin protein synthesis in the fat body. While in holometabolous insects, such as *Aedes aegypti* (Dittmann et al., 1989; Raikhel and Lea, 1990) and *Drosophila melanogaster* (Soller et al., 1999), there is an evolutionary shift from the use of JH to the use of ecdysteroids, secreted by the ovary, as vitellogenic hormone. The vitellogenin genes have been cloned and characterized in several insect species. Cockroaches have become classical model to study JH dependent vitellogenesis (Engelmann, 1983).

Most of the studies have been down on *Leucophaea maderae*, *Blattella germanica* and *Blaberus discoidalis* (Keely et al., 1985; Martin et al., 1995, 1996; Comas et al., 2001; Engelmann, 2002; Engelmann and Mala, 2005). However, the two vitellogenin genes *Vg1* and *Vg2* were previously cloned and sequenced from the female fat body of the american cockroach, *P. americana* (Tufail et al., 2000, 2001), there is no information available about their genomic copy numbers and / or their regulatory expression levels.

The present work shows, for the first time, that both *Vg1* and *Vg2* are represented each by a single copy in the genome of the *P. americana*. In addition, the *in vivo* developmental expression levels of *Vg1* and *Vg2* have been studied using real time PCR and RT-PCR. The correlation between the two genes expression levels and the JH and 20E hormones titers during oogenesis was disclosed.

The present work will open the way for better understanding of the vitellogenin gene hormonal regulation on the molecular level.

2. MATERIALS AND METHODS

2.1. Animals

Colonies of *P.americana* were maintained under constant dark, feeding on artificial diet MF (Oriental Yeast Co.Ltd.Tokyo) and water ad libitum. White female roaches were collected daily and kept in groups. The fat body was collected in different developmental time, from day 1 to day 12 for studying the expression profile.

2.2. Southern Blot Analysis

The genomic DNA was isolated from female fat body using Mammalian Genomic DNA Kit (Sigma, St.Louis, MO). Purity of the nucleic acid was assessed using the A260/A280 ratio (Sambrook and Russell, 2001). For Southern blotting, 10 µg of genomic DNA was digested with the six enzymes: *ApaI*, *BamHI*, *NheI*, *NotI*, *PvuII* and *XbaI* (Takara, Kyoto, Japan). The digested DNA was ethanol precipitated and separated on a 0.9 % agarose gel, denatured and transferred into nylon membrane (Immobilon-Ny+, Millipore, Billerica, MA, USA) by capillary blotting. The membrane was prehybridized in ExpressHyb Hybridization solution (BD Bioscience, USA) for 30 min at 60°C and hybridized for 1 h at the same temperature and in the same buffer with the radiolabelled probe. The cDNA probes for each gene were generated by digestion of *Vg1* (2013 to 2278bp) using *EcoR I* and *Vg2* (3663 to 3967 bp) using *EcoRI* and *EcoRV* from the cDNA library clones. Megaprime-TM DNA labeling systems (Amersham, USA) was used to obtain ³²P-labelled probe. The hybridized membranes were washed twice in wash solution I (2 X SSC, 0.05% SDS) for 20 min each at room temperature and twice in wash solution II (2X SSC, 0.1% SDS) for 20 min each at 50°C. For signal detection, the hybridized membranes were exposed to BAS1000 (Fuji Photo Film, Tokyo, Japan) and analyzed.

2.3. RT-PCR and Real Time PCR

Fat bodies from adult females were collected during days of oogenesis (days 1 to 12), frozen immediately in liquid nitrogen and kept at -80°C until use. Total RNA was extracted by Isogen reagent (Nacalai tesque, Kyoto, Japan) according to the manufacturer's instructions. Two µg DNase-treated total RNA were reverse transcribed using ReverTra Ace (Toyobo Co., Osaka, Japan) and poly dT primer according to the manufacture's instructions. Primers set (Table 1) for vitellogenin genes, *Vg1* and *Vg2* in addition to, the housekeeping gene, actin, were designed using Primer Express Version 1.5 (Applied Biosystem) and tested to ensure amplification of a single band using RT-PCR. The PCR amplification cycles were 94°C for 2 min , then 30 cycles of 94°C for 30 sec, then 56°C for 30 sec and 72°C for 2 min with 3 min extension . As a reference, the cDNA was subject to the same PCR conditions with specific primers to Actin . The amplified fragments were sequenced for conformation. The RT-PCR products were run on 2 % agarose gel, which were stained with ethidium bromide, and photographed with the AE-6910 gel imaging system (ATTO,Tokyo, Japan).

Table 1. List of primers

Experiment Name	Primer Name	Sequence and its position on cDNA
Real-time-PCR	Vg1-F	CTGCGTCCAGAGGATTCTGAATTG (5216 to 5238 bp)
	Vg1-R	ATTATGCAAATCAGAATTAGAGCA (5324 to 5346 bp)
	Vg2-F	ATATTCCACCTCCGAGGCCACCAA (917 to 940 bp)
	Vg2-R	CTGCTATCTGAGTTACTTTCTAG (1015 to 1037 bp)
	Actin-F	TGACTGAGCGTGGTTACAGC (330 to 394 bp)
	Actin-R	CAGGAAGGAAGGTTGGAACA (534 to 553 bp)

The real time PCR amplification was performed on ABI 700 Sequence Detection System (Applied Biosystem) by using SYBR GreenI MasterMix Plus (Eurogentec, Seraing, Belgium).

Each reaction was contained 2.5µl of cDNA template and 50 nM primers in a final volume of 25 µl. Cycling parameters were 95°C for 10 min to activate DNA polymerase, and then 40 cycles of 95°C for 15 sec and 60°C for 1min.

Melting curves were drawn using Dissociation Curves software (Applied Biosystems) to ensure that only a single product was amplified for each gene. Each cDNA was run four times and the average used for analysis. The value of Vg1, 2 mRNA were measure relative to those of Actin as housekeeping gene at each time point and expressed as a relative (Vg1 or 2/ Actin) ratio.

Statistical Analysis

The changes in Vg1 and Vg2 mRNA expression were analyzed by one-way ANOVA followed by Student t-test using Origin program (SAS Institute Inc. USA).

3. RESULTS

3.1. Analysis of Vitellogenin Genes Copy Number

To estimate the copy number of Vg1 and Vg2 genes in the genome of *P. americana*, Southern blot analysis was carried out. The digested genomic DNA was hybridized with ³²P-labeled cDNA probes as described in the material and methods. Southern blot analysis revealed one hybridization band in genomic DNA digested with *ApaI*, *NheI*, *NotI* for Vg1 and digested with *BamHI*, *PvuII* and *XbaI* enzymes for Vg2 (Figure 1). This result indicated that the Vg1 and Vg2 are represented in the cockroach genome by a single copy gene.

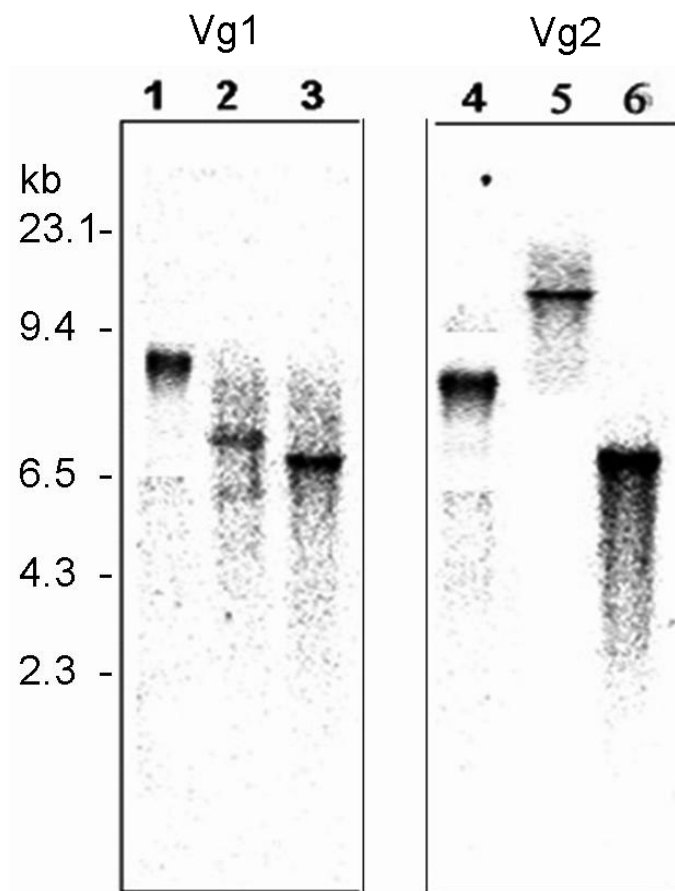


Figure 1. Detection of genomic copy number of *P.americana* vitellogenin genes. Genomic DNA digested with *ApaI*, *NheI*, *NotI* enzymes for *Vg1* and with *BamHI*, *PvuII* and *Xba I* enzymes for *Vg2* (1-6) was electrophoresed and hybridized with ^{32} P-labelled *Vg1* and *Vg2* cDNA probes respectively. The size of markers is indicated on the left.

3.2. Expression Analysis of Vitellogenin Genes (*Vg1* and *Vg2*):

We examined the *in vivo* developmental expression patterns of both vitellogenin genes semi-quantitatively with RT-PCR technique, using total RNAs isolated from female fat body during the oogenesis. We checked the amplified products length on 2 % agarose gel and was found to be 130 bp, 124 bp, and 135 bp corresponding to *Vg1*, *Vg2* , and Actin respectively (Fig. 2A).

To determine the relative concentration of *Vg1* and *Vg2* expression related to the reference gene Actin, during female oogenesis, we applied real time PCR using SYBR-green I reagent. Detection of *Vg1* transcript started at day 3 while *Vg2* transcripts started more earlier at day 2 of female previtellogenic stage. Both genes transcripts gradually increased and peak at day 7 and day 10 of vitellogenic stage. No transcripts were detected during days 1, 9 and 12. The relative concentration of *Vg2* transcript was higher than that of *Vg1* (Fig. 2B).

4. DISCUSSION

The fat body is the main site of vitellogenin synthesis in the majority of insect. The present study showed that *Vg1* and *Vg2* genes of the American cockroach *P. americana* are represented in the genome each by a single copy as revealed by southern blot analysis (Figure 1). Similar results were found in the cockroach *L. madarea* for *Vg* genes (Tufail et al., 2007). However the *Vg* genes have been cloned and characterized from other insects such as *Aedes aegypti*, *Bombyx mori*, *Athalia rosa* and *Apis mellifera*, the representation of such genes in the genome of these insects have not been studied (reviewed in Tufail and Takeda, 2008).

In order to confirm the usefulness of *Vg1* and *Vg2* designated primers used in the present work for the real time PCR, we carried out RT-PCR experiment. The amplified products from RT-PCR reaction using primers set for each gene showed a single band of the expected size for each gene (Figure 2A), suggesting that the designated primers are useful for using in the real time PCR experiment.

In the present work, the expression profile of *Vg1* mRNA is found to be similar to that of *Vg2*. However, the expression level of *Vg2* is found to be consistently higher than that of *Vg1* in the fat body cells (Figure 2B). Both expression profile and level of *Vg1* and *Vg2* are found to be synchronous and up-regulated by the hemolymph JH titer (Weaver and Partt, 1977) during this developmental period. Interestingly, *Vg2* transcript was first detected at day 2, while *Vg1* transcript was detected later at day 3, suggesting that the two *Vg* genes might have different hormonal regulatory mechanism. On the other hand, neither *Vg1* nor *Vg2* transcripts were detected during days 1, 9 and 12, suggesting that the transcription of both genes are inhibited by the hemolymph free ecdystroid (Weaver et al., 1984) secreted from the ovary of the female *P.americana* during these days. Our results are consistent with those obtained for *L. madarea* (Engelmann and Mala, 2005); *Blattella germanica* (Comas et al., 2001) and *Heliothis virescens* (Ramasway et al., 1997) in which JH is capable of fast induction of *Vg* mRNA expression in the female fat body. However, in gypsy moth, *Lymantria dispar*, high JH titer can even suppress the transcription of *Vg* gene (Fescemyer et al., 1992). On the other hand, in dipterans such as house fly, *Musca domestica* and the mosquito *Aedes aegypti*, 20E is the chief hormone involved in *Vg* transcription (Raikhel et al., 2002; Agui et al., 1991), while in the hawk moth, *Manduca sexta*, JH is only needed to finalized oocyte growth (Satyanarayana et al., 1994).

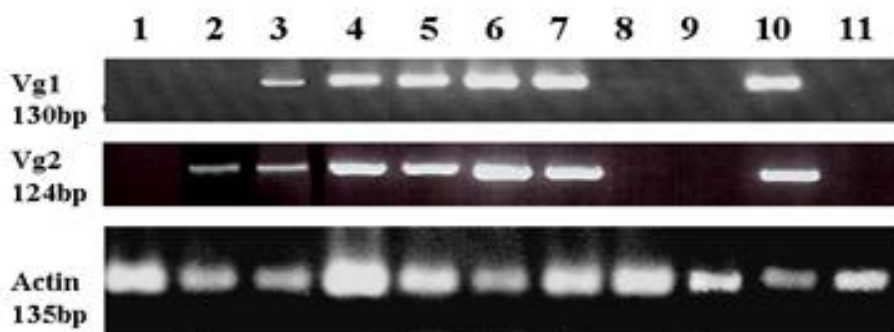


Figure 2A. RT-PCR analysis of *Vg1* and *Vg2* genes of adult female fat body of *P.americana* from the first day of emergence.

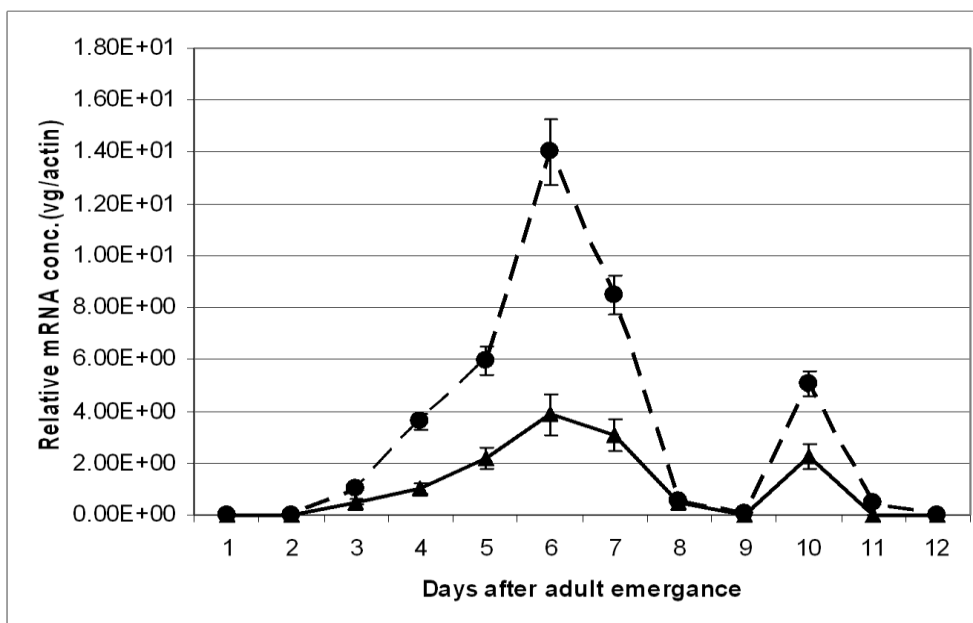


Figure 2B. Relative expression analysis of *Vg1* and *Vg2* mRNA during days from adult female emergence of the cockroach *P. americana*. Vitellogenin genes expressions were analyzed by Real-time PCR and were plotted relative to Actin gene expression. The dash line is *Vg2* while the smooth one is *Vg1*. Error bars represent SEM from four independent measurements.

In conclusion, the present work represent, for the first time, the gene copy number for both *Vg1* and *Vg2* genes, which are the yolk proteins precursor's genes in the fat body of female *P. americana*, are presented in the genome by a single copy for each gene. The *Vg2* is the predominant vitellogenin gene expressed in the *P. americana* fat body cells during vitellogenesis and its expression precede that of *Vg1*. Both expression profile and level of *Vg1* and *Vg2* are found to be synchronous and up-regulated by the hemolymph JH titer and suppressed by hemolymph ecdysteroid titer during vitellogenesis. The difference in the *Vg1* and *Vg2* gene expression level detected in the present study reflects the responsive differences of these two genes to hemolymph JH titer. The present study will pave the way toward investigating the JH responsive elements that regulate the expression of these genes.

REFERENCES

- Agui, N., Shimada, T., Yomino, S. 1991. Hormonal control of vitellogenin messenger RNA in the male and female housefly, *Musca domestica*. *J. Insect Physiol.*, 37, 383-390.
- Belles, X. 1998. Endocrine effectors in insect vitellogenesis, in *Recent Advance in Arthroid Endocrinology*. (Eds. G.M. Coast and S. G. Webster), Cambridge University Press, Cambridge, pp.71-90.
- Belles, X. 2005. Reproductive Biology of Invertebrates, Progress in Vitellogenesis, vol.12B. *Vitellogenesis directed by juvenile hormone*. In: Raikhel, A., Sappington, T.W. (Eds), Sci Publishers, Enfield NH, pp, 157-198.

- Borst, D.W., Eskew, M.R., Wagner, S.J., Shores, K., Hunter, J., Luker, L., Hatle, J.D., Hecht, L.B. 2000. Quantification of juvenile hormone III, vitellogenin, and vitellogenin mRNA during the oviposition cycle of their lubber grasshopper. *Insect Biochem. Molec.Biol.*, 30, 813-819.
- Comas, D., Piulachs, M.D., Belles, X. 2001. Induction of vitellogenin gene transcription in vitro by juvenile hormone in *Blattella germanica*. *Mol. Cell. Endocrin.*, 183, 93-100.
- Dittmann, F., Kogan, P.H., Hagedorn, H.H. 1989. Ploidy levels and DNA synthesis in fat body cells of the adult mosquito, *Aedes aegypti*. The role of juvenile hormone, *Arch. Insect Biochem. Physiol.*, 12,133-134.
- Delisle, J., Cusson, M. 1999. Juvenile hormone biosynthesis, oocyte growth and vitellogenin accumulation in *Choristoneura fumiferana* and *C. rosaceana*: a comparative study. *J. Insect Physiol.*, 45, 515-523.
- della-Cippa, G., Engelmann, F. 1984. Juvenile hormone regulation of phospholipid synthesis in the endoplasmic reticulum on vitellogenic fat body cells from *Lecuphaea maderae*. *Insect Biochem.*, 14, 27-36.
- Don-Wheeler, G., Engelmann, F. 1997. The biosynthesis and processing of vitellogenin in the fat bodies of females and males of the cockroach *Leucophaea maderae*. *Insect Biochem. Molec. Biol.*, 27,901-918.
- Edwards, G.C., Braun, R.P., Wyatt, G.R. 1993. Induction of vitellogenin synthesis in *Locusta migratoria* by the juvenile hormone analog, pyriproxyfen. *J. Insect Physiol.*, 39, 609-614.
- Engelmann, F. 1983. Vitellogenesis controlled by juvenile hormone in *Endocrinology of insect* (Eds. R.G.H. Downer and H. Laufer), Vol1, Alan R. Liss, Inc., New York, pp. 259-270.
- Engelmann, F. 2002. Ecdysteroids, juvenile hormone and vitellogenesis in the cockroach *Leucophaea maderae*. *J. Insect Sci.*, 2 (20), 1-8.
- Engelmann, F., Mala, J. 2005. The cockroach *Leucophaea maderae* needs more than juvenile hormone, vitellogenin and reserves to make a yolky egg. *J. Insect Physiol.*, 51(4), 465-72.
- Fescemyer, H.W., Masler, R.E., Davis, R.E., Kelly, T.J. 1992. Vitellogenin synthesis in female larvae of the gypsy moth, *Lymantria dispar* (L.): Suppression by juvenile hormone. *Comp. Biochem. Physiol.*, 103B, 533-542.
- Hagedorn, H. 1985. *The role of ecdysteroids in reproduction in Comprehensive Insect Physiology, Biochemistry and Pharmacology*, (Eds. G.A. Kerkut and L.I. Gilbert) Vol.8, Oxford, Pergamon Press, pp. 205-261.
- Imboden, H., Konig, R., Ott, P., Lustig, A., Kampfer, V., and Lanzrein, B. 1987. Characterization of the native vitellogenin and vitellin of the cockroach *Nauphoeta cinerea* and comparison with other species. *Insect Bioch.*, 17, 353-365.
- Irvine, D.J., Brasch, K. 1981. The influence of juvenile hormone on Polyploidy and vitellogenesis in the fat body of *Locusta migratoria*. *Gen. Comp.Endocrinol.*, 45, 91-99.
- Ishizaka, S., Bhaskaran, G., Dahm, K.H. 1989. Juvenile hormone production and ovarian maturation in adult *Manduca sexta*, in *regulation of Insect Reproduction* (Eds. M. Tonner, T. Soldan and B. Bennetova), Vol. 4, Institute of Entomology, Czechoslovak Academy of Science, Prague., pp.49-57.
- Keeley, L., and McKercher, S.R. 1985. Endocrine regulation of ovarian maturation in the cockroach *Blaberus discoidalis*. *Comp. Biochem. Physiol.*, 80A, 115-121.

- Martin, D., Piulachs, M.D., Belles, X. 1995. Pattern of haemolymph vitellogenin and ovarian vitellin in the germane cockroach, and the role of juvenile hormone. *Physiol. Entomol.*, 20, 59-65.
- Martin, D., Piulachs, M.D., Bellés, X. 1996. Inhibition of vitellogenin production by allatostatin in the German cockroach. *Mol. Cell. Endocrinol.*, 121(2):191-6.
- Nair, K.K., Chen, T.T., Wyatt, G.R. 1981. Juvenile hormone stimulated polyplody in adult locust fat body. *Dev. Biol.*, 81, 356-360.
- Raikhel, A.S., Lea, A.O. 1990. Control of follicular epithelium development and vitellin envelope formation in the mosquito: Role of juvenile hormone and 20-hydroxyecdysone. *Tissue Cell.*, 23, 577-591.
- Raikhel, A.S., Dhadialla, T.S. 1992. Accumulation of yolk proteins in insect oocytes. *Annual Review of Entomology.*, 37, 217-251.
- Raikhel, A.S., Kokoza, V.A., Zhu, J.S., Martin, D., Wang, S.F., Li, C., Sun, G.Q., Ahmed, A., Dittme, N., Attardo, G. 2002. Molecular biology of mosquito vitellogenesis: From basic studies to genetic engineering of antipathogen immunity. *Insect Biochem. Mol. Biol.*, 32, 1275-1286.
- Raikhel, A.S., Brown, M.R., Belles, X. 2004. Comprehensive Molecular Insect Science. In Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), *Hormonal control of reproductive processes*, vol.3. Elsevier, Oxford, pp 433-491.
- Ramaswamy, S.B., Shu, S.Q., Park, Y.I., Zeng, F.R. 1997. Dynamics of juvenile hormone-mediated gonadotropism in the Lepidoptera. *Arch. Insect Biochem. Physiol.* 35, 539-558.
- Sambrook, J., Russell, D.W. 2001. Molecular cloning A Laboratory Manual. *Cold Spring Harbor Laboratory Press*. Third Edition. Cold Spring Harbor, New York.
- Satyanarayana, K., Bradfield, J.Y., Bhaskaran, J., Dahm, K.H. 1994. Stimulation of vitellogenin production by methoprene in prepupae and pupae of *Manduca sexta*. *Arch. Insect Biochem. Physiol.*, 25, 21-37.
- Soller, M., Bownes, M., Kubli, E. 1999. Control of oocyte maturation in sexually mature *Drosophila* females. *Dev. Biol.*, 208, 337-351.
- Sappington, T.W., Raikhel, A.S. 1998. Molecular characteristics of insect vitellogenin receptors. *Insect Biochem. Molec. Biol.*, 28, 277-300.
- Tufail, M., Hatakeyama M., Oishi K., Takeda M. 2000. Cloning of vitellogenin cDNA of the American cockroach, *Periplaneta americana* (Dictyoptera), and its structural and expression analyses. *Arch Insect Biochem Physiol.*, 45(1), 37-46.
- Tufail, M., Hatakeyama M., Takeda M. 2001. Molecular evidence for two vitellogenin genes and processing of vitellogenins in the American cockroach, *Periplaneta americana*. *Arch Insect Biochem Physiol.*, 48 (2), 72-80.
- Tufail, M., Bembenek, J., Elgendy, A.M., Takeda, M. 2007. Evidence for two vitellogenin-related genes in *Leucophaea maderae*: the protein primary structure and its processing. *Arch. Insect Biochem. Physiol.*, 66, 190-203.
- Tufail, M., Takeda, M. 2008. Molecular characteristics of insect vitellogenins. *J. Insect Physiol.*, 54, 1447-1458.
- Weaver, R.J., Partt, G.E. 1977. The effect of enforced virginity and subsequent mating on the activity of the corpus allatum of *Periplaneta americana* measured in vitro, as related to changes in the rate of ovarian maturation. *Physiol. Ent.*, 2, 59-76.
- Weaver, R.J., Strambi, A., Strambi C. 1984. The significance of free ecdysteroids in the haemolymph of adult cockroaches. *J. Insect Physiol.*, 30, 705-711.

- Wyatt, G.R., Davey, K.G. 1996. Cellular and molecular actions of juvenile hormone. III Roles of juvenile hormone in adult insects. *Adv. Insect Physiol.*, 26, 1-155.
- Zhang, J., Saleh, D., Wyatt, G.R. 1996. Juvenile hormone regulation of an insect gene: a specific transcription factor and a DNA response element. *Mol. Cell. Endocrin.*, 122, 15-20.

LECTIN BINDING PATTERN OF INTESTINAL GOBLET CELLS IN HORSE, PIG AND RABBIT

***J. M. Galotta^{*1,2}, S. G. Márquez^{1,3}, C. N. Zanuzzi^{4,5}, E. J. Gimeno⁴,
E. L. Portiansky⁴ and C. G. Barbeito^{4,5}***

¹Laboratorio Anatomía. Facultad Ciencias Agrarias,
Pontificia Universidad Católica Argentina

²Cátedra Anatomía. Facultad Ciencias Veterinarias,
Universidad de Buenos Aires

³Ciclo Básico Común, Universidad de Buenos Aires

⁴Instituto de Patología. Facultad Ciencias Veterinarias,
Universidad Nacional de La Plata.

⁵Cátedra de Histología y Embriología. Facultad Ciencias Veterinarias,
Universidad Nacional de La Plata

ABSTRACT

The carbohydrate expression of goblet cells in the intestine of pigs, rabbits and horses was characterized using lectin histochemistry. Paraffin embedded intestinal slices were incubated with the following biotinylated lectins: Con-A, WGA, DBA, SBA, PNA, RCA-1 and UEA-1. Except for some rectal goblet cells present in pigs and horses, no reactivity was found to Con-A. On the contrary, reactivity to DBA, WGA and RCA-1 was heterogeneous in all the intestinal sections of the studied species. PNA reactivity was absent in the small intestine with the exception of few positive cells present in the horse. DBA did not bind to colonic goblet cells in the pig, while SBA did not bind the same cells in pigs and horses. RCA-1 staining was negative in the large intestine of the horse. The lectin binding with UEA-1 differed among different intestinal sections and species, remaining unstained in the small intestine of rabbits, colon of horses and rectum of pigs.

Our results led us to conclude that the lectin binding pattern of the intestinal goblet cells is characteristic of each animal species and shows variations related with the intestinal segment analyzed.

Keywords: Goblet cells, lectin histochemistry, intestine.

* Postal address: Jorge Galotta, Facultad Ciencias Agrarias. Pontificia Universidad Católica Argentina. Freire 183. 1426 Ciudad Autónoma de Buenos Aires. Argentina. Tel/fax (54 11) 4552-2711 / 2721 / 2724; jgalotta@uca.edu.ar

INTRODUCTION

Different cell types are present in mammal intestinal epithelium: stem cells, enterocytes, goblet cells (GC), enteroendocrine cells and Paneth cells. Stem cells give rise to all the epithelial above mentioned lineages [1, 2]. Progenitor cells that express Math1 become goblet, enteroendocrine or Paneth cells. On the other hand, Math1-negative progenitors give rise to enterocytes. Further differentiation of the secretory lineage into goblet, enteroendocrine or Paneth cells requires the expression of additional factors [3].

GC, also known as mucus secretor-producer cells, not only are found in the intestine, but also they can be seen in other organs. The characteristic “goblet” shape, due to the accumulation of premucin droplets produced when the cell is actively functioning, distinguishes them from other epithelial cells such as the enterocytes. They produce viscous, dense and elastic mucus that protects and lubricates the epithelial surface. Besides this mechanical function, it participates in diverse digestive and immunologic activities. The mucus is blend into the glycocalix at the brush border surface and becomes a viscous coat that tends to trap molecules that are next to the apical membrane of enterocytes. Moreover, this mucous layer functions as a barrier to avoid pathogen microorganism colonization [4].

The premucin granules can not be always identified using traditional histochemical techniques, being necessary other specific stains, such as the PAS and Alcian histochemical techniques, and lectin histochemistry (LHC).

Lectins are heterogenous plant derived proteins and glycoproteins which bind specifically but not immunologically to specific terminal carbohydrate residues [5]. Taking into account this property, the lectin histochemistry technique is used to localize, identify and distinguish differences in cellular carbohydrate sequences with high sensibility [6]. Furthermore, it is more specific than other traditional techniques for carbohydrate identification [7]. Our goal was to identify GC using LHC to compare the terminal oligosaccharide premucin droplets as well as the glycocalix mucins in the horse, pig and rabbit.

MATERIALS AND METHODS

Clinically healthy 6 seven-years old cross-bred male horses, 6 six-month old hybrid commercial male pigs, and 6 three-month old New Zealand male rabbits, all of them slaughtered in commercial abattoirs were used.

Five segments of the middle portion of the jejunum, ileum, colon and rectum were fixed with neutral buffered 10% formalin. After overnight fixation, tissues were processed for paraffin embedding and sectioned at 5 μ m. After dewaxing, sections were treated with 0.3% H₂O₂ in methanol for 30 min at room temperature, rinsed several times in 0.01 M PBS, and treated with 0.1% bovine serum albumin in PBS for 15 min. Sections were then incubated during 2 h at room temperature with the biotinylated lectins.

The seven used lectins (Lectin Kit BK 1000, Vector Laboratories, Inc., Burlingame, CA, USA) with different carbohydrate specificity were the following: Con-A (*Concanavalia ensiformes*), specifically binding α -D-Man and α -D-Glc); DBA (*Dolichus biflorus*, with binding specificity to α -D-GalNAc); SBA (*Glicine max*), binding specificity to α -D-GalNAc, β -D-galNAc and α and β -Gal); PNA (*Arachis hypogea*, that specifically binds β -D-

Gal and (1-3) GalNAc); RCA-1 (*Ricinus communis*-1, binding specificity β -D-Gal and α -D-Gal); UEA-1 (*Ulex europaeus*-1, binding specificity α -L-Fuc), WGA (*Triticum vulgaris*, binding specificity α -D GlcNAc and NeuNAc). The optimal lectin concentration was 30 μ g/ml in PBS for all lectins, except for PNA (10 μ g/ml). The slides were incubated with an avidin-biotin-peroxidase complex (ABC®, Vector Laboratories, Inc) during 45 min. The horseradish peroxidase was activated by the diaminobenzidine commercial kit (Dako, Carpinteria, CA, USA) incubated for 1 to 2 min. Specimens were rinsed in distilled water, dehydrated with graded ethanol, cleared in xylene and mounted in Permount (Fisher Scientific International, Hampton, NH, USA). Negative controls included exposure to horseradish-peroxidase and substrate medium without lectin. The intensity of lectin binding was subjectively scored as follows: (-) none, (+) weakly positive, (++) positive, (+++) strongly positive.

RESULTS

According to our observation GC were identified in each organ of the three studied species but with differences in the lectin binding in each case. Table 1 summarises the intensity of the lectin binding pattern of the three studied species.

Table 1. Lectin histochemical staining pattern observed in horse, pig and rabbit

Lectin	Jejunum			Ileum		
	Horse	Pig	Rabbit	Horse	Pig	Rabbit
Con A	-	-	-	-	-	-
WGA	+	+ / ++ ¹	+	++	++	+ / - ¹
DBA	++	+	+ ¹	++ ¹	+	+ ¹
SBA	++ ¹	++	++	++	++ / +++	+ ¹
PNA	++ ¹	-	-	++	-	-
RCA - 1	+++	+	++	++	+ / ++	+ ¹
UEA - 1	+++	++	-	++	+++	+ / -

Lectin	Colon			Rectum		
	Horse	Pig	Rabbit	Horse	Pig	Rabbit
Con A	-	-	-	+ ¹	+	+ ¹
WGA	-	++	+	+	+ / ++	+ ¹
DBA	+ ²	-	+	++ ^{1,2}	+	++ ^{1,3}
SBA	-	-	+	+ ²	++	++ ¹
PNA	-	-	-	-	-	-
RCA - 1	+ / -	+++	+	-	++	+ ¹
UEA - 1	-	+++	+ / ++	++	+ / ++	++ ¹

(-) no staining, (+) weakly positive, (++) positive, (+++) strongly positive.

¹. Heterogeneous staining (some GC were positive while some others were negative); 2. GC located at the surface of the intestinal crypts (closed to the lumen of the organ); 3 GC located at the bottom of the crypts.

In the jejunum of the horse, pig and rabbit WGA stained GC localised at the bottom of the crypt, showing a heterogeneous staining pattern. On the contrary, GC in the horse jejunum showed a moderate staining with DBA (Figure 1A), whereas it was weak in the other two species. SBA also moderately stained the cells in the three studied species.

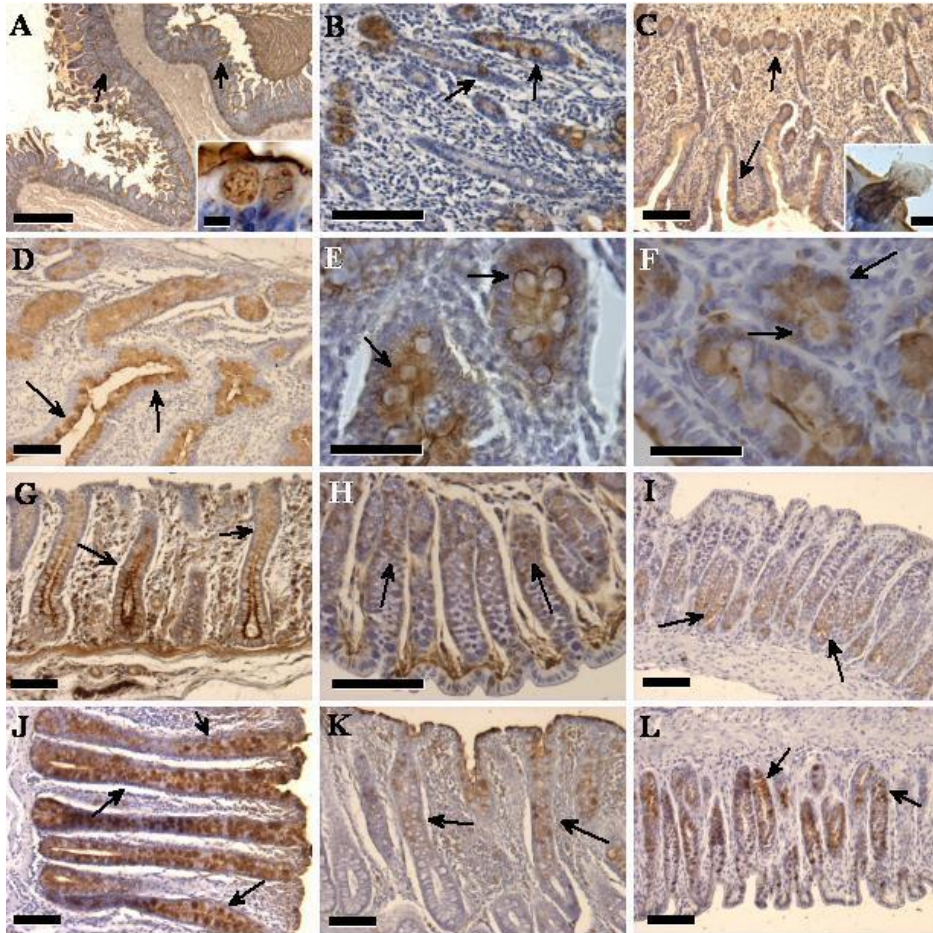


Figure 1. Histological aspect of GC stained with different lectins in the studied organs. A. Horse jejunum incubated with DBA. Positive GC are seen in the superficial epithelium (arrows). Bar = 1 mm. Inset: detail of a GC at higher magnification. Bar = 10 μ m. Heterogeneous staining of secretory granules can be observed. B. Horse ileum incubated with SBA. Arrows point to GC strongly labelled. Bar = 100 μ m. C. Horse ileum incubated with WGA. Strongly stained GC can be observed in both, superficial and cryptal epithelium (arrows). Bar = 100 μ m. Inset: Exocytosis in a GC. Bar = 10 μ m. D. Pig ileum incubated with SBA. Strong staining of GC is observed (arrows). Bar = 100 μ m. E. Pig ileum incubated with UEA-I. GC membrane secretory granules are strongly stained (arrows). Bar = 50 μ m. F. Rabbit ileum incubated with DBA. Heterogeneity in the binding pattern of GC is observed (arrows). Bar = 50 μ m. G. Pig colon incubated with RCA-I. Superficial GC are moderately stained (arrows). Bar = 100 μ m. H. Rabbit colon incubated with RCA-I. Heterogeneous and weak staining of GC is observed (arrows). Bar = 100 μ m. I. Rabbit colon incubated with SBA. Intestinal crypt GC are strongly stained (arrows). Bar = 100 μ m. J. Horse rectum incubated with UEA-I. Strongly stained GC can be observed in cryptal epithelium (arrows). Bar = 100 μ m. K. Pig rectum incubated with SBA. Few GC located in the middle of the crypt are moderately labelled (arrows). Bar = 100 μ m. L. Rabbit rectum incubated with SBA. Positive reacting cryptal GC is observed (arrows). Bar = 100 μ m.

PNA bound GC in the horse but not in the other species. Some GC in horse jejunum showed a finely granular pattern. Horse jejunum GC were strongly positive to RCA-1. In this species membrane secretory vesicles and their content were delineated. On the contrary, in the rabbit and pig, few cells were stained and, in general, the lectin binding intensity was weak. The majority of the stained cells in rabbit jejunum were near to the surface. Finally, UEA-1 strongly stained cells in horse jejunum while was negative for rabbit tissues.

In the ileum, all the lectins except Con-A moderately stained GC of the horse (Figure 1B,C). Positive GC in horse ileum was found at the bottom and at the surface of the intestinal crypts. On the contrary, rabbit cells showed a very weakly staining for all the lectins except for PNA, which was negative. Pig GC were intensely stained with SBA and UEA-1 (Figure 1D,E), moderately with WGA, and weakly to moderate with DBA and RCA-1. PNA was also negative in porcine GC. Membrane secretory vesicles of pigs' ileal GC were heterogeneously labelled.

WGA labelling was heterogeneous in the colon of the three studied species. No GC was stained in the horse. However, in rabbits they were weakly stained and in pigs they showed the highest labelling intensity. Superficial GC positively reacted with DBA in all the species. SBA did not bind to GC neither in the horse colon nor in the porcine tissues. On the contrary, the labelling was heterogeneous in rabbits, since some cells were positive while others remained unstained. RCA-1 showed variable reactivity to GC in the colon of the studied species, being weak in horse but strongly positive in pigs. UEA-1 labelling was negative in horse GC. It strongly bounded to porcine GC and only to some cells in rabbits.

Rectal GC was weakly stained by Con-A in the three considered species but WGA, DBA and SBA moderately bound them. Rectal DBA positive rabbit cells were observed close to the bottom of the crypts. RCA-1 stained porcine and rabbit GC. UEA-1 showed a similar binding pattern in the three species, although porcine rectal cells showed the highest labelling intensity.

DISCUSSION

The lectin binding pattern observed in the present work showed great variation, which not only depended on the specie, but also on the intestinal segment studied.

Con-A lectin did not bind to GC, except for some scattered rectal cells in pigs and horses. This result could mean that mannosa and glucosa terminal residues are not present in the mucin of most of these cells. This is in accordance with previous studies, as it occurs in the human [8] and bovine ileum [9]. Our results agree with the ones described by More [10] in porcine ileum, but they differ from the studies published by Chae [11], Sohn [12], and Choi [13]. The latter authors observed positive reaction with Con A. These differences could be attributed to age, because Chae worked with three weeks-old pigs, while Sohn and Choi used one week-old animals. Age related lectin binding affinity differences in porcine intestine had been previously mentioned by others authors [14-16]. Con-A binding to rectal goblet cells was heterogeneous in all the studied species, and this finding is similar to that reported by Freeman [17] in the rat colon. The existence of some Con-A positive cells could be interpreted as regional differences in the mucus composition. This fact could be related to functional differences in each sector. Glucose and mannose residues were identified in human

large intestinal mucus [18, 19] and our results seem to confirm that in most species only this sector has oligosaccharides with these terminal residues.

Regarding WGA, there was a heterogeneous lectin binding pattern both, among species and different intestinal sections. In horses the lectin binding was weak, except in the ileum, where the staining was stronger. In pigs the lectin staining was more homogenous in all the analysed segments. A similar strong staining pattern for WGA was found in porcine ileum [11], but our binding pattern differed from that described in young animals, in which no reactivity was observed [12]. Concerning these data, we conclude that there exist age-related differences in porcine GC lectin binding. When comparing these results to those reported in other species, bovine ileum showed high reactivity as well [9]. In the small and large intestinal WGA lectin binding of GC was similar to that described in humans [18]. None of the studied species showed marked variations along the crypt-villus axis as it was described in the man by Vecchi [8].

GC of all the intestinal segments were heterogeneously labelled with DBA, but no GC were stained in porcine colon. Positive cells were observed at the bottom of the large intestinal crypts in rabbit, in accordance with the data reported by Doebrn [20] in mouse caecum. On the other hand, there are many positive cells in the horse large intestine. A similar positive lectin-binding pattern had been previously described in human and mouse GC [21], and in bovine [9]. DBA staining was negative both in the ileum [12] and jejunum [13] of one week old-pigs. Nevertheless, accordingly to Chae [11] DBA is positive in the ileum of three week old-pigs. We also observed positive DBA reactivity but in adult pigs. The changes described could be attributed to mucin composition variations in the small intestinal goblet cells at early stage of life. This composition could remain partially invariable until adulthood.

SBA bound to all GC except for those in the colon of horses and pigs. A coincidence was found between the positive reaction in adult pig small intestine and young pigs [11-13], but a different pattern was observed in pigs during weaning state by Jaeger [14]. A strong staining was also present in GC of bovine ileum [9]. Except for the results published by Jaeger, it can be deduced that ileal GC mucus has high content of galactose and N-acetilgalactosamine in all species. The positive reaction observed in rabbit colon GC is similar to that found in rat [17] and mouse caecum [20].

PNA did not bind to GC in the small bowel, except for some cells in the horse. This is in accordance to the negative results found in man [8, 18, 20]; and in the bovine [9]. Controversial results were found in pigs. Our results are similar to those published by Chae [11] and Choi [13], but differ from those reported by Riganti [23] and Sohn [12], which included pigs of different ages. Even though positive GC were observed in rat colon [17] most of the results indicate that no positive PNA labelling was found in the large bowel in any of the three studied species. Such differences could be attributed to variations in the sensitivity and sensibility of the used techniques. Positive PNA reacting cells were observed deep in the crypts [24] while those cells located in the middle and superficial regions of the crypt remained negatives. We did not found such regional and differential distribution.

RCA-1 heterogeneously bound GC, except for those present in equine rectum, most of rat distal colon [17] and human jejunum [8]. However, other authors indicate that small and large bowel GC are RCA-1 positives [18]. The positive labelling of porcine GC has been previously mentioned [11-14].

UEA-1 intensely bound to most of the GC except for those in rabbit small bowel and horse colon. Furthermore, ileal GC staining was less intense in the upper part of the crypt than

in its bottom. The same pattern was described in human jejunum [8] and it can be attributed to cellular differentiation mechanisms. Nevertheless, Freeman [17] observed the inverse pattern: higher reactivity at the bottom of the rat colonic crypts. Our results are coincident with those published by Riganti [23] in the same specie. Negative reaction of small bowel GC was also described in bovines [9].

The present work contributes to establish the lectin histochemical pattern of intestinal GC in different mammal species. This lectin binding pattern is modified by different factors, such as age [14-16, 25] and diet [10, 26, 27]. Mucin glycoconjugate complexes of each intestinal sector studied may suffer variations, which depend not only on the chemical composition but also on the mucus layer thickness [28]. These physiological considerations are relevant since they show notorious modifications on the glycoconjugates as a result of dietary variations (changes from suckling state to the balanced diet of adult animal) [29, 30].

Differences in the staining pattern could be due to modifications in the mucin glycosylation pattern [31] generated by a change in the expression of the gene that codify to glycosyltransferases or glycosidases and secondarily they could depend on differential gene expression for the protein portion of them [32]. This glycosylation does not reach complete maturation after the weaning period [33]. Enterocytes, but not Paneth cells, changes in the lectin binding pattern consequently to a increment of a transcription regulator: the vitamin D [Zanuzzi et al., unpublished, 34].

Regional and inter specific differences of the mucin could reflect the interaction between epithelium-microorganisms or host-parasite and the effects of various cytokines produced by the host in response to such interaction [35-37]. These interactions could modify the glycolisation pattern, as a result of a coevolutive process [38]. The same regional differences have been previously mentioned when studying mucin secretory cells present in other organs. For example, a differential lectin histochemical pattern was found throughout the reproductive tract of heifers [39] and in porcine salpinx [40].

This study indicates that the lectin binding pattern of GC is characteristic of each animal species studied and there are variations on the carbohydrate pattern in different intestinal regions and in different regions along the crypt-villus axis. The information generated could be a relevant biological tool to compare, determine and analyze possible glycoside changes of the intestinal mucin under diverse conditions, as dietary changes and pathological stages.

ACKNOWLEDGMENTS

The technical assistance of Ruben Mario is gratefully acknowledged. CNZ, ELP, EJG and CGB are research career members of Argentinean Research Council (CONICET).

REFERENCES

- [1] Cheng, H; Leblond, C. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. *Am. J. Anat.* 1974; 141: 537-561.

- [2] de Santa Bárbara, P; van Den Brink, GR; Roberts DJ. Development and differentiation of the intestinal epithelium. *Cell Mol. Life Sci.* 2003 60: 1-11.
- [3] Yang, Q; Bermingham, NA; Finegold, MJ; Zoghbi, HY Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* 2001; 294: 2155-2158.
- [4] Walker, WA. Development of the Intestinal Mucosal Barrier. *Journal of Pediatric Gastroenterology and Nutrition* 2002; 34: S33-S39.
- [5] Spicer, SS; Schulte, BA. Diversity of cell glycoconjugates shown histochemically: a perspective. *J Histochem Cytochem* 1994; 44: 82-84.
- [6] Danguy, A; Decaestecker, C; Genten, F; Salmon, I; Kiss, R. *Applications of Lectins and Neoglycoconjugates in Histology and Pathology Acta Anat* 1998; 161: 206-218.
- [7] Gimeno, EJ; Barbeito, CG. Glicobiología, una nueva dimensión para el estudio de la biología y la patología. *Anales Academia de Agronomía y Veterinaria* 2004; 58: 1-34.
- [8] Vecchi, M; Torgano, G; Monti, M; Berti, E; Agape, D; Primignani, M; Ronchi, G; de Franchis, R. Evaluation of structural and secretory glycoconjugates in normal human jejunum by means of lectin histochemistry. *Histochemistry* 1987; 86: 359-364.
- [9] Massone, AR; Itagaki, S; Koi, K; Gimeno EJ. Lectin histochemical Study on Normal and Paratuberculosis - Affected Bovine Ileum. *J. Vet. Med. Sci.* 1991; 53(4): 761-763.
- [10] More, J; Fioramonti, J; Benazet, F; Bueno, L. Histochemical characterization of glycoproteins present in jejunal and colonic goblet cells of pigs on different diets. A biopsy study using chemical methods and peroxidase-labelled lectins. *Histochemistry* 1987; 87(2): 189-194.
- [11] Chae, C. Lectin Histochemical Characteristics of the Epithelial Surface of Ileal Peyer's Patches in 3-Week-Old pigs. *J. Vet. Med. Sci.* 1997; 59(10): 931-934.
- [12] Sohn, Y-S; Chae, C. Lectin-binding Capacity of Glycoconjugates in Escherichia coli Infected Porcine Lower Small Intestines. *J. Vet. Med. Sci.* 2000; 62(5): 543-547.
- [13] Choi, B-Y; Sohn, Y-S; Choi, C; Chae, C. Lectin Histochemistry for Glycoconjugates in the Small Intestines of Piglets Naturally Infected with Isospora suis. *J. Vet. Med. Sci.* 2003; 65(3): 389-392.
- [14] Jaeger, LA; Lamar, CH; Turek, JJ. Lectin binding to small intestinal goblet cells of newborn, suckling and weaned pigs. *Am. J. Vet. Res.* 1989; 50(11): 1984-1987.
- [15] Gelberg, H; Whiteley, H; Ballard, G; Scott, J; Kuhlenschmidt, M. Temporal lectin histochemical characterization of porcine small intestine. *Am. J. Vet. Res.* 1992; 53(10): 1873-1880.
- [16] Chae, C; Lee, YS. Age-related lectin histochemical changes in the porcine small intestine. *J. Vet. Med. Sci.* 1995; 57(5): 883-889.
- [17] Freeman, HJ; Reubenlotan, FRCP; Kim, YS. Application of lectins for Detection of Goblet Cell Glycoconjugate Differences in Proximal and Distal Colon of the Rat. *Laboratory Investigation* 1980; 42(4): 405-412.
- [18] Fischer, J; Klein, PJ; Vierbuchen, M; Fischer, R; Uhlenbruck, G. Histochemical and Biochemical Characterization of Glycoprotein Components. Normal Gastric Mucosa, Intestinal Metaplasia and Gastric Cancers with Lectins. In: Walter de Gruyter and Co, editor. *Lectins*. Volumen III. Berlin - New York. 1983; 157-166.
- [19] Madrid, JF; Aparicio, R; Saez, FJ; Hernández, F. Lectin cytochemical characterization of the N- and O- linked oligosaccharides in the human rectum. *Histochemical Journal* 2000; 32(5): 281-289.

- [20] Doebrn, S; Reipohl, W; Lierse, W; Romarnuk, K; Young, W. Developmental Changes in the Distribution of Cecal Lectin-Binding Sites of Balb-c Mice. *Acta anat* 1992; 145: 296-301.
- [21] Danguy, A; Kiss, R; Pasteels, J-L. Lectins in Histochemistry. *Biological Structures and Morphogenesis* 1988; 1(3): 93-106.
- [22] Coggi, G; Dell Orto, P; Bonoldi, E; Doi, P; Viale, G. Lectins in diagnostic Pathology. In: Walter de Gruyter and Co, Editor. *Lectins*. Volumen III. Berlin - New York. 1983; 87-103.
- [23] Riganti, JG; Massone, AR; Quiroga, MJ; Armocida, AD; Perfumo, CJ. Lectin histochemical Binding Profile of Ileum Samples from Pigs affected with Proliferative Enteropathies. *Rev. Med. Vet.* 1997; 78: 69-72.
- [24] Stoward, PJ; Spicer, SS; Miller, RL. Histochemical reactivity of peanut lectin-horseradish peroxidase conjugate. *J. Histochem. Cytochem.* 1980; 28: 979-990.
- [25] Caldero, J; Campo, E; Calomarde, X; Torra, M. Distribution and changes of glycoconjugates in rat colonic mucosa during development. A histochemical study using lectins. *Histochemistry* 1988; 90(4): 261-270.
- [26] Brown, PJ; Poulsen, SS; Wells, M. Serine protease immunohistochemistry and lectin histochemistry in the small intestine of weaned and unweaned pigs. *Res. Vet. Sci.* 1991; 50(1): 1-5.
- [27] Conour, JE; Ganessunker, D; Tappenden, KA; Donovan, SM; Gaskins, HR. Acidomucin goblet cell expansion induced by parenteral nutrition in the small intestine of piglets. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2002; 283(5): 1185-1196.
- [28] Szentkuti, L; Lorenz, K. The thickness of the mucus layer in different segments of the rat intestine. *Histochemical Journal* 1995; 27(6): 466-472.
- [29] Bolte, G; Knauss, M; Metzendorf, I; Ster, M. Postnatal maturation of rat small intestinal brush border membranes correlates with increase in food protein binding capacity. *Dig. Dis. Sci.* 1998; 43(1): 148-155.
- [30] Pácha, J Development of Intestinal Transport Function in Mammals. *Physiological Reviews* 2000; 80(4): 1633-1667.
- [31] Jass, JR; Walsh, MD Altered mucin expression in the gastrointestinal tract: a review. *J. Cell Mol. Med.* 2001; 5(3): 327-351.
- [32] Buisine, MP; Devisme, L; Savidge, TC; Gespach, C; Gosselin, B; Porchet, N; Auberta, JP. Mucin gene expression in human embryonic and fetal intestine. *Gut* 1998; 43: 519-524.
- [33] Biol-N'garagba, M-C; Louisot, P. Regulation of the intestinal glycoprotein glycosylation during postnatal development: role of hormonal and nutritional factors. *Biochimie* 2003; 85: 331-352.
- [34] Zanuzzi, CN; Fontana, PA; Barbeito, CG; Portiansky, EL; Gimeno, EJ. Paneth cells: histochemical and morphometric study in control and *Solanum glaucophyllum* intoxicated rabbits. *European Journal of Histochemistry* 2008; 52(2): 93-100.
- [35] Deplancke, B; Gaskins, HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am. J. Clin. Nut.* 2001; 73(6): 1131-1141.
- [36] Sharma, R; Schumacher, U; Ronaasen, V; Coates, M. Rat intestinal mucosal responses to a microbial flora and different diets. *Gut* 1995; 36: 209-214.

-
- [37] Hicks, SJ; Theodoropoulos, G; Carrington, SD; Corfield, AP. The role of mucins in host-parasite interactions. Part I protozoan parasites. *Parasitol Today* 2000; 16: 476-481.
 - [38] Freitas, M; Axelsson, L-G; Cayuela, Ch; Midtvedt, T; Trugnan, G. Microbial-host interactions specifically control the glycosylation pattern in intestinal mouse mucosa. *Histochemistry and Cell Biology* 2002; 118(2): 149-161.
 - [39] Cobo, ER; Campero, CM; Gimeno, EJ; Barbeito, CG. Lectin Binding Patterns and Immunohistochemical Antigen Detection in the Genitalia of *Tritrichomonas* foetus-infected Heifers. *J. Comp. Path.* 2004; 131: 127-134.
 - [40] Santana, FJF; Nascimento, EF; Gimeno, EJ; Barbeito, CG. Cyclic Related and Pathological Changes in the Lectin-binding Sites on the Swine Oviduct. *Reprod. Dom. Anim.* 2004; 39: 1-6.

ULTRASTRUCTURAL ASPECTS AND FREQUENCY OF CILIATED FIBROBLASTS IN THE PERIODONTAL LIGAMENT OF INCISOR TOOTH OF RATS SUBMITTED TO DIFFERENT ERUPTION CONDITIONS

***Pedro Duarte Novaes^{1*}, Simone de Cássia Barbosa¹,
Nádia Fayez Omar¹, Juliana dos Santos Neves¹, Eliene Aparecida
Orsini Narvaes¹, Maria Albertina de Miranda Soares² and
José Rosa Gomes²***

¹Departamento de Morfologia. Faculdade de Odontologia de Piracicaba,
UNICAMP. Piracicaba, SP, P.O. Box 52, Piracicaba, SP-13.414-900 Brasil

²Departamento de Biologia Estrutural Molecular e Genética, UEPG. Ponta Grossa, Pr,
Brasil. Avenue Carlos Cavalcanti, 4748 Ponta Grossa Pr 84030-900, Brasil

ABSTRACT

The relationship between ciliated fibroblast found in the periodontal ligament of rodent incisor teeth and eruption rate was studied under different eruption conditions, using an estimation of eruption rate and ultra structural aspects of primary cilia. Inferior left incisor teeth were cut for 24 days to increase the rate of eruption. During this period, the rate of eruption doubled; however, after this period, the number and frequency of ciliated fibroblasts was not altered. This lack of change does not necessarily signify that ciliated fibroblast do not have a direct or indirect role in the process of eruption, as suggested by the characteristics of the periodontal ligament structure and emerging functions for the primary cilium found in other tissues.

INTRODUCTION

Cilia are cell surface, membrane bound projections that have a centriolar origin. In epithelial cells, the cilium axoneme consists of nine mobile doublet microtubules surrounding two central microtubules (9+2). The microtubules make contact with each other via dynein

* Email: novaes@fop.unicamp.br; phone/fax (55)19 2106335.

arms and by employing ATP recruitment that enables millions of cilia to execute their movement on the cell surface. Whilst some cells have multiple cilia, others cells, such as condrocytes and fibroblasts, have a single non-motile cilium that is denominated the primary or solitary cilium (Munger, 1958; Latta *et al.*, 1961; Wilson and Mc Whorter, 1966; Bari and Sorensen, 1965; Scherft and Daems, 1967; Dahl, 1967; Dingemans, 1969; Rash *et al.*, 1969; Federman and Nichols, 1974; Webber and Lee, 1975; Park *et al.*, 1988; Goranova and Chaldakov, 1989; Stubblefield and Brinkley, 1966; Wheatley, 1969, 1971, 1972; Archer and Wheatley, 1971). Generally, solitary cilia do not present a central pair of microtubules (9+0) and do not have dynein arms (for review, see Satir and Christensen, 2007). Solitary cilia can still present great variation in the number and arrangement of their microtubules, as first observed by Zimmerman (1898) in the rabbit renal tubule and in the human seminal vesicle.

During the early 1960s, some studies postulated that solitary cilia could participate in sensorial functions (Barnes, 1961; Sorokin, 1962) and many recent studies have reported the presence of specific receptors and ion channel proteins in the cilia membrane that could be involved in signaling pathways comparable to those described for motile 9+2 cilia (Moran and Rowley, 1983; Pazourot and Witman, 2003; Praetorius and Spring, 2005; Satir and Christensen, 2007). In contrast, Latta *et al.* (1961) suggested the solitary cilia to be a vestige of evolution. As such, the exactly role of solitary cilia in tissues, currently, remains unknown.

The presence of solitary cilia has been reported in fibroblasts in cell culture (Stubblefield and Brinkley, 1966; Wheatley, 1969, 1971, 1972; Archer and Wheatley, 1971) and in oral tissues. Solitary cilia have also been observed in tooth pulp fibroblasts in dogs (Kubota, 1977) and in the periodontal ligament of the rodent incisor teeth presenting continuous growth and eruption (Bertseen *et al.*, 1975; Barbosa, 1999).

The eruption rate of incisor teeth is approximately 0.5mm/day under normal condition, however, when the tooth is shortened, eruption increases at double the rate of normal conditions (Michaeli *et al.*, 1983). It has been hypothesized that teeth shortening produces a hypofunctional state and it is one of the best model for studies regarding eruption processes. On the other hand, in periodontal ligaments of incisor teeth, Barbosa *et al.* (2003) demonstrated that the number of ciliated fibroblasts is higher, compared to dermal and gingival fibroblasts and suggested that these increased numbers of ciliated cells could be associated with the metabolic activity of the periodontal ligament of the incisor teeth and, consequently, with the eruption process. Thus, to assess whether there is any relationships between the number and frequency of ciliated fibroblasts in periodontal ligaments and the eruption process, in this study the inferior incisor teeth were submitted to different eruption conditions.

MATERIAL AND METHODS

Fourteen male Wistar rats (150-200g body weight) were divided into two groups (7 rats/group). The left lower incisor tooth was shortened at the intergingival level using a diamond high-speed rotating instrument every two days for a total of 24 days to produce a hypofunctional eruption condition, while the right lower incisor tooth remained in a hyperfunctional eruption condition. The control group, had all left lower incisor tooth maintained in a normal functional eruption condition.

In the intact teeth, a reference mark was made with a diamond high-speed rotating instrument and the eruption rates were measured using a calibrated grid under a microscope eyepiece at 10x magnification every two days during the entire experimental period. The reference distances (mm) from the gingival margin to the marks made on the right teeth (normal and hyper functional groups), up to the end of the left incisor teeth (hypofunctional groups) were recorded. For these manipulations, all animals were lightly anaesthetized with halothane. The eruption rates were analyzed using the *Student t* test, at $P < 0.05$ level.

At the end of the experimental period, all rats received an intramuscular injection of Ketamine (80 mg/Kg) and Xylazine (8 mg/Kg), (Francotar and 2% Verbaxyl, respectively, Virbac, Brazil). An intracardiac perfusion with a solution of 2.5% glutaraldehyde in 0.1M phosphate buffer was carried out for 15 minutes. The mandibles were dissected and separated into hemi-mandibles, which were then immersed in 2.5% glutaraldehyde in 0.1M phosphate buffer for a further 3 hours at room temperature. Hemi-mandibles were then maintained in 5% EDTA in 0.1M phosphate buffer, pH 7.4, at room temperature, for 55 days with daily changes of solution. After complete demineralization, hemi-mandibles were cut transversely, taking the third molar as a reference and the samples were post fixed in osmium 1% tetroxide in 0.1M phosphate buffer, pH 7.4, for 3 hours, and washed in 0.1M phosphate buffer, before dehydrating with acetone and embedding in araldite resin. Ultra fine sections (60 to 90 nm) were obtained and contrasted with uranyl acetate and lead citrate. Sections were observed in an EM 10 electron microscopic (Carl Zeiss) at amagnification of 16 000X. For each animal and treatment, 600 fibroblasts were counted, totaling 4200 fibroblasts per group.

To the estimate of the real number of ciliated fibroblasts were used the ligament attached to the tooth as well as on the bone. The estimative were made by counting the number of cilia, basal bodies, basal body pairs, centriolar complexes, basal bodies/centriole pairs, centriole pairs and the basal bodies associated or not with the cilia. The real number of ciliated fibroblasts was estimated according to Beertsen *et al.* (1975), where the % = number of basal bodies-centriole pairs/number of centriole pairs + number of basal bodies-centriole pairs X 100; Archer and Wheatley (1971) and Wheatley (1972) where the % = number of cilia + basal bodies/centriolar complexes X 100; Wheatley (1971) where the % = basal bodies with cilia/basal bodies-centrioles (F) X 100 and Wheatley (1971) where the % = basal bodies with cilia + basal bodies without cilia/basal bodies-centrioles X 100. All data obtained were submitted to analysis of variance (ANOVA) and Tukey test at $P < 0.05$ level.

RESULTS

The eruption rate during the experimental period is demonstrated in Figure 1. A significant increase in eruption rate was detected during the period by the *Student t* test ($p < 0.05$).

Figure 2 (A and B) shows the ultrastructural aspects of the solitary cilia of fibroblasts in the periodontal ligaments of incisor teeth showed that the cilia extended from the distal end of the basal bodies (BB) and projected from the cell surface 3 shows and an accumulation of the filamentous proteins, which are denominated as basal foot (BF) or satellite were usually observed at the lateral side of the basal body. A single centriole (C) was generally connected

to the basal body by a cross-banded structure (CBE). This figure also shows a membrane invagination, (MI) which is deep laterally to the cilium.

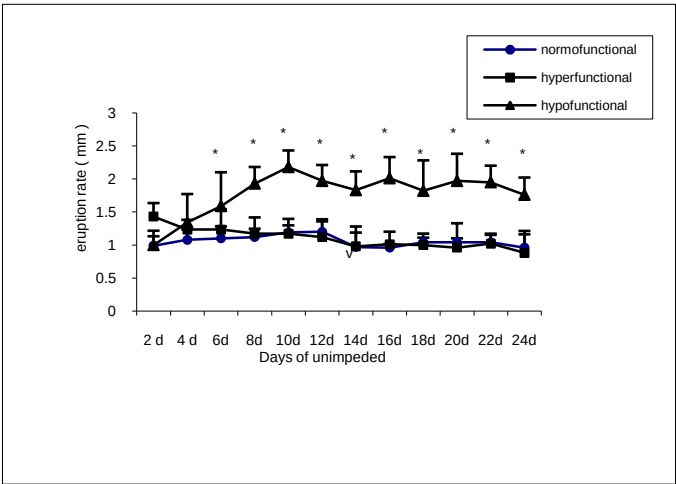


Figure 1. Eruption rate (mm) of the left inferior incisor tooth. Mean ± DP. (*P*<0.05)

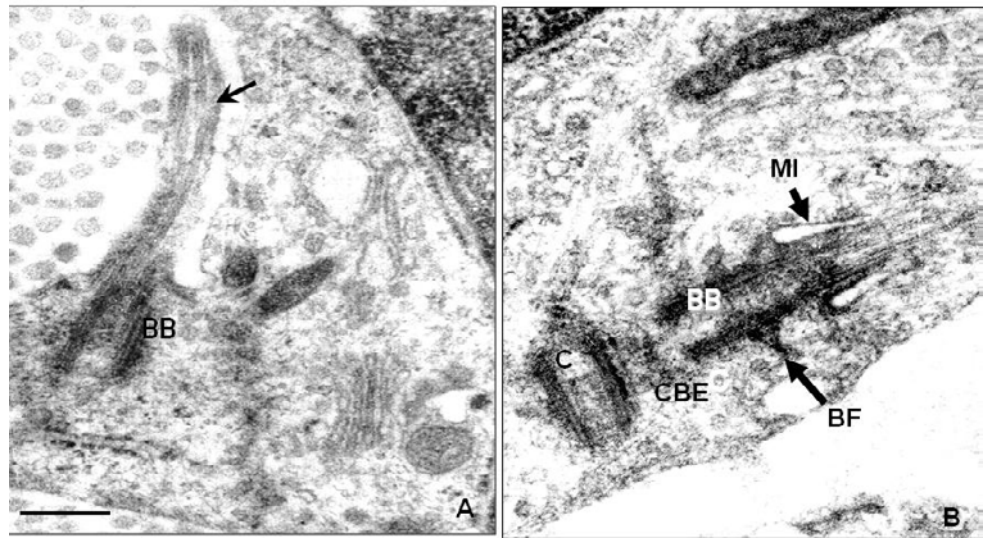


Figure 2. An electron micrograph of a longitudinal section of a solitary cilium of fibroblast in the periodontal ligament of the inferior incisor tooth. A-The solitary cilium extends from the distal end of the basal body (BB) and projected from the apical cell surface of the cell (arrow). B- present the body basal (BB), an membrane invagination (MI) and a single centriole (C) connected to basal body by a cross-banded structure (CBE). Scale bar = 0.28 μ m.

The solitary cilia in cross section predominantly presented a 9+0 microtubular array, as shown in Figure 3. However, other microtubular solitary cilia arrangements were observed in cross sections; these included 8+2, 6+2, and 4+0 and are also shown in Figure 3.

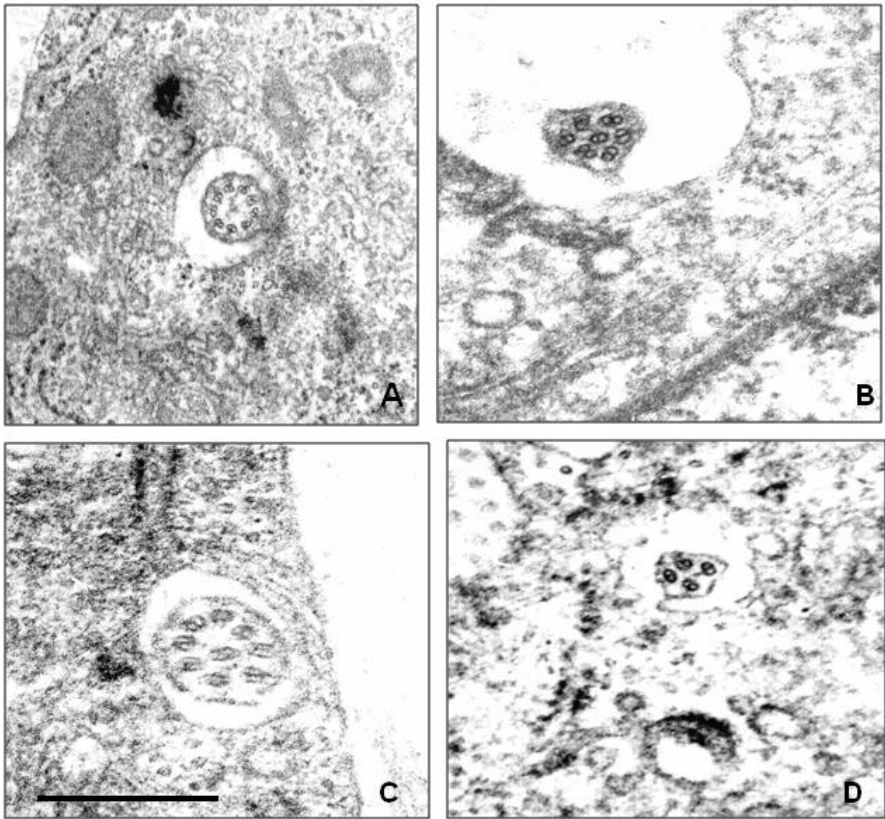


Figure 3. Electron micrographs of a cross section of a solitary cilium in the periodontal ligament showing irregular microtubular arrangements. A.9+0; B.6+2;C.8+2 and D.4+0. Scale bar=0,28µm.

The structural parameters of the ciliated fibroblasts of the periodontal ligament obtained for each eruption condition were established by different methods and are demonstrated in Table 1 and in Table 2.

Table 1. Number of cellular structures used to estimate the frequency of ciliated fibroblast in the periodontal ligament of the lower tooth of mouse submitted to different eruption conditions (Hypo: function diminished of the left lower incisor tooth by shortened; Hyper: right inferior lower tooth with hyperfunction maintained; Normal: left lower incisor tooth with normal function). The data was submitted to analysis of variance (ANOVA) and Tukey test at level of $P<0.05$

Eruption conditions	A- Centriolar complex	B Basal Body	C Solitary cilia	D Basal body pair	E Centriole pair	F Basal/body centriole	G Basal Body with cilia	H Basal body without c
Hyper	255	26	113	18	16	66	15	26
Hypo	293	19	121	16	22	59	20	19
Normal	268	11	121	14.5	13.5	59	21	11

Table 2. Frequency and estimative (%) of the real number of the ciliated fibroblast in the periodontal ligament of the lower tooth of mouse submitted to different eruption conditions (Hypo: function diminished of the left lower incisor tooth by shortened; Hyper: right lower incisor tooth with hyperfunction maintained; Normal: left lower incisor tooth with normal function) calculated by distinct methods. The data was submitted to analysis of variance (ANOVA) and Tukey test at level of $P < 0.05$

Real number estimative of the ciliated fibroblast					
Eruption condition	Cilia frequency	Beertsen <i>et al.</i> (1975)	Archer & Wheatley (1971,1972)	Wheatley (1971)	Wheatley (1971)
Hyper	2.8	53	54.5	22	62
Hypo	3.3	42	48	34	66
Normal	3.3	52	49	36	54

*Beertsen *et al.* (1975) where the % = number of basal bodies-centrioles pairs (D)/number of centrioles pairs (E) + number of basal bodies-centrioles pairs (D) X 100; **Archer & Wheatley (1971) and Wheatley (1972) where the % = number of cilia (C) + basal bodies (B)/centriolar complex(A) X 100; ***Wheatley (1971) where the % = basal bodies with cilia (G)/basal bodies-centrioles (F) X 100. ****Wheatley (1971) where the % = basal bodies with cilia (G) + basal bodies without cilia (H)/basal bodies- centrioles (F) X 100.

Statistical analysis did not demonstrate significant differences between the frequency and number of ciliated fibroblasts of the incisor tooth periodontal ligaments that were submitted to different eruption conditions and underwent different techniques. However, the frequency of ciliated fibroblasts for this tissue was relatively high, even during normal eruption.

DISCUSSION

Rodent incisor teeth constitute an appropriate model for studies regarding the eruption process, since they demonstrate continuous growth and eruption. Thus, this characteristic allows their submission to different eruption experimental conditions. To increase the eruption rate of the incisor tooth, the shortening treatment has been shown to be the most efficient methodology. This technique increases the eruption rate by twofold, compared to impeded conditions (Michaeli *et al.*, 1983, Gerlach *et al.*, 2000). In the present study, this method was found to increase the eruption rate throughout the experimental period (Figure 1). It is also believed that the continuous growth and eruption of the incisor tooth produced by the shortening treatment leads to a higher turnover of the periodontal ligament components of the extracellular matrix and cells. It has been reported that glycosylaminoglycan is increased during accelerated eruption processes (Kirkham *et al.*, 1993). On the other hand, the fibroblast is the principal cell found in the periodontal ligament. Ciliated fibroblasts found in the periodontal ligament under normal eruption conditions correspond to 70% (Beertsen *et al.*, 1975) of the periodontal ligament. This assessment was confirmed by Barbosa *et al.* (2003), in a study that demonstrated that the frequency of ciliated fibroblasts was higher in periodontal ligaments compared to other connective tissues. From these findings, we hypothesized that the acceleration of the eruption process by the shortening treatment could alter the frequency and number of fibroblasts solitary cilia. For this evaluation, four methods used elsewhere (Beertsen *et al.*, 1975; Archer and Wheatley, 1971; Wheatley, 1971 and

Wheatley, 1971) were applied. These methods are based on different ultrastructural parameters observed in fibroblasts. Thus, Table 2 presents an altered frequency of ciliated fibroblast following the shortening treatment, however, when results were submitted to statistical analyses, no significant differences in the frequency and numbers of ciliated fibroblasts were found for incisor teeth submitted to different eruption conditions.

These analyses also demonstrate that any of the methods used could have been applied to this study. Since the methods used, herein, are based on different and independent ultrastructural aspects, the results of the study were not found to be significant. Results of the study show that the frequency and number of fibroblasts remain constant after the period of shortening treatment. Thus, we cannot affirm that the ciliated fibroblasts, which correspond to 70% of the cells of the ligament, did not play any role in gradual eruption processes since, unfortunately, this approach was not carried out in this study. We suggest that possibly, there is a moment during the increase in eruption, as shown in Figure 1, in which the fibroblast frequency also increases, however this must be further studied to estimate the frequency of fibroblast each two days along of shortened treatment. However, the frequency of ciliated fibroblasts in the periodontal ligament, as shown by different methods, was considered to be higher in all eruptions conditions, as previously demonstrated by Barbosa et al (1999).

The ultrastructural aspects of the solitary cilia found in cells of the periodontal ligament of rat incisor teeth, submitted to different eruption conditions, were similar to those of previous literature descriptions, i.e., presence of a basal foot or satellite in the lateral side of the basal body and a centriole connected to the basal body by a cross-banded structure (Hagiwara *et al.*, 2002); however, no changes were detected in the hypofunctional eruption condition. A microtubular array of 9+0 was also observed in this study, although other variations in numbers of axoneme microtubules were also observed (8+2, 6+2 and 4+0). These variations in numbers of axoneme microtubules of the solitary cilia have already been reported in the dental pulp by Kubota (1977), who suggested a relationship between the microtubules of axoneme and the cell origin; e.g., epithelial cells have a normal pattern array of 9+2, while tissues of mesenchymal origin could present distinct microtubular arrays. This may occur due to the fact that the periodontal ligament has a mesenchymal origin. A variation in the number of microtubules was also observed in the solitary cilia of the periodontal ligament fibroblast (figure 3); however, whilst this finding is of interest, the exact role of these differences in microtubular arrangement in the solitary cilium in different tissues is unclear and worthy of further study.

Chailley *et al.* (1989) and Boisvieux-Ulrich *et al.* (1990) proposed that the basal foot may promote the movement of cilia by rotation or rearrangement of the basal body in cells of the epithelial oviduct. Another functional explanation for the basal foot in solitary cilia was proposed by Tenkova and Chaldakov (1988) and Poole *et al.* (1997). These authors suggested that the cilia could determine cell polarity, since the basal foot is linked to the Golgi apparatus and may be necessary for exocytosis. In other tissues, a potential role of solitary cilia in sensorial functions has been suggested (Poole *et al.*, 1985; Roth *et al.*, 1988; Schwartz *et al.*, 1997; Wheatley, 1995; Wiederhold, 1976). Solitary cilia were suggested by Munges (1958), Goranova and Chaldakov (1989) to resemble a chemoreceptor after they observed this organelle in cells of the Langerhans island and in smooth muscular fibers in the uterus, respectively. Maraspin and Boccabella (1971) demonstrated that the production of solitary cilia was inhibited by estradiol in the muscular uterus, indicating a chemoreceptor function.

Studies published recently by Anderson et al (2008) and Whitfield (2008) reported mechanical and chemical functions for solitary cilia with a focus on skeletal biology. The solitary cilia deliver intracellular signals, such as Ca^{2+} produced by compression of cartilage fluid that triggers a cascade of intracellular events that include appropriate gene activations to maintain the strength of bone and cartilage. Furthermore, authors demonstrated that the polycystic protein has a mechanically sensitive function and serves as a flow sensor in cultured renal epithelium.

Although this study did not aim to evaluate the periodontal ligament as a tissue submitted to constant masticator forces, it is possible that solitary cilia could have a specific sensorial function in maintaining the homeostasis of the extra cellular environment of the tooth during the eruption process. Solitary cilia may directly influence this process, since the periodontal ligament seems to demonstrate a higher and constant turnover of its components, including cells, collagen fibers and collagenases involved in the remodeling process. It is possible that solitary cilia of the periodontal ligament could also be involved in mechanical or chemical functional responses to the masticator forces that occur during dental function. Such a hypothesis opens new perspectives as regards the functional properties of the periodontal ligament and appropriate studies concerning solitary cilia function in this mechanism are required.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Mrs Eliene A. O. Narvaes and FAPESP for financial support. Experiments described were accomplished according to the current animal welfare laws of Brazil.

REFERENCES

- Anderson CT, Castillo AB, Brugmann SA, Helms JA, Jacobs CR, Stearns T. 2008. Primary cilia: cellular sensors for the skeleton. *Anat. Rec. (Hoboken)*. 291(9):1074-8.
- Archer FL, Wheatley DN. 1971. Cilia in cell-cultured suresfibroblasts. Incidence in mitotic and post-mitotic BHK 21 – C13 fibroblast. *J. Anat.* 109, 277-292.
- Bari WA, Sorensen GD. 1965. Ciliated cells the spleen of adult rats. *Anat. Rec.* 152, 481-6.
- Barnes BG. 1961. Ciliated secretory cells in the pars distalis of the mouse hypofisis. *J. Ultrastruct. Res.* 5, 453-67.
- Barbosa SC, Merzel J, Novaes PD. 2003. Ciliated fibroblasts in mouse connective tissues: a comparison between methods for estimating their frequency. *J. Submicrosc. Cytol. Pathol.* 35(1), 83-89.
- Beertsen W, Everts V, Houtkooper JM. 1975. Frequency of occurrence and position of cilia in fibroblasts of the periodontal ligament of de incisor. *Cell Tiss. Res.* 163, 415-31.
- Boisvieux-Ulrich E, Laine MC, Sandoz D. 1990. Cytochalasin D inhibits basal body migration and ciliary elongation in quail oviduct epithelium. *Cell Tissue Res* 259(3), 443-54.

- Chailley B, Nicolas G, Laine MC. 1989. Organization of actin microfilaments in the apical border of oviduct ciliated cells. *Biol. Cell* 67(1), 81-90.
- Dahl HA. 1967. On the cilium cell relationship in the adenohypophysis of the mouse. *Z. Zellforsch* 83, 169-77.
- Dingemans KP. 1969. The relation between cilia and mitoses in the mouse adenohypophysis. *J Cell Biol.* 43,361-367.
- Federman M, Nichols GJr. 1974. Bone cell cilia: vestigial or functional organelles? *Calcif Tiss. Res.* 17, 81-85.
- Gerlach RF, Toledo DB, Novaes PD, Line SRP, Merzel. 2002. Alveolar bone remodeling pattern of the rat incisor under different functional conditions as shown by minocycline administration. *Archs Oral. Biol.*, 47:203-209.
- Goranova V, Chaldakov GN. 1989. Ciliated fibroblasts and smooth muscle cells in the rat uterus. *Experientia* 46, 488-9.
- Hagiwara H, Harada S, Maeda S, Aoki T, Ohwada N, Takata K. 2002. Ultrastructural and immunohistochemical study of the basal apparatus of solitary cilia in the human oviduct epithelium. *J. Anat.* 200, 89-96.
- Kirkham J, Robinson C, Phull JK, Shore RC, BKB Moxham BJ, Berkovivitz BKB. 1993. The effect of rate of eruption on periodontal ligament glycosylaminoglycan content and enamel formation in the rat incisor. *Cell tissue Res.* 274:413-419.
- Kubota K. 1977. Comparative study of the single cilium of dental pulp cells. *J. Nihon Univ. Sch Dent* 11,11-5.
- Latta H, Maunsbach AB, Madden SC. 1961. Cilia in different of the rat nephron. *J Biophy Biochem. Cytol.* 11,248-52.
- Maraspin LE, Boccabella AV. 1971. Solitary cilia in endometrial fibroblasts. *J. Reprod Fert* 25,343-7.
- Munger BL. 1958. A light and electron microscopic study of celular differentiation in the islets of mouse. *Amer. J. Anat.* 103,275-311.
- Moran DT, Rowley JC. 1983. The structure and function of sensory cilia. *J. Submiscroc. Cytol.* 15(1),157-162.
- Michaeli Y, Steigman S, Yitschaky M, Weinreb M. 1983. The response of the fibroblast population in the periodontal ligament of the rat incisors to altered eruption rates. *J. Periodontal Res.* 21(1): 86-94.
- Park P, Manabe S, Ohno T. 1988. Incidence and ultrastructure of rudimentary cilia in benign and malignant peripheral nerve tumores. *Ultrastruct. Path* 12, 407-418.
- Pazour GJ, Witman GB. 2003. The vertebrate primary cilium is a sensory organelle. *Curr. Opin. Cell Biol.* 15(1),105-10.
- Poole CA, Flint MH, Beaumont BW. 1985. Analysis of the morphology and function of primary cilia in connective tissue: A cellular cybernetic probe? *Cell Moti* 5, 175-94.
- Poole CA, Jensen CG, Snyder JA, Gray CG, Hermanutz VL, Wheatley DN. 1997. Confocal analysis of primary cilia structure and colocalization with the Golgi apparatus in chondrocytes and aortic smooth muscle cells. *Cell Biol. Interna* 21, 483-494.
- Prateorius HA, Spring KR. 2005. A physiological view of the primary cilium. *Annu. Rev. Physiol.* 67,515-529.
- Rash JE, Shay JW, Bieseke JJ. 1969. Cilia in cardiac differentiation. *J. Ultrastruct. Res.* 29,470-84.

- Roth KE, Rieder CL, Bowser SS. 1988. Flexible-substratum technique for viewing cells from the side: some in viv properties of primary (9+0) cilia in cultured kidney epithelia. *J. Cell Sci.* 89,457-66.
- Satir P, Christensen ST. 2007. Overview of structure and function of mammalian cilia. *Annu. Rev. Physiol.* 69,377-400.
- Scherft JP, Daems WT. 1967. Single cilia in chondrocytes. *J. Ultrastruct Res.* 19,546-55.
- Schwartz EA, Leonard ML, Bizios R, Bowser SS. 1997. Analysis and modeling of the primary cilium beding response to fluid shear. *Am. J. Physiol. (Renal Physiology)* 272,132-38.
- Sorokin S. 1962. Centrioles and the formation of rudimentary cilia by fibroblasts and smooth muscle cell. (1962) *J. Cel Biol* .15,363-77.
- Stubblefield E, Brinkley BR. 1966. Cilia formation in chinese hamster fibroblasts in vitro as a response to colcemid treatment. *J. Cell Biol.* 30,647-52.
- Tenkova T, Chaldakov GN. 1988 Golgi-cilium complex in rabbit ciliary process cells. *Cell Struct. Fun.* 13(5), 455-8.
- Zimmerman KW. 1989. Beiträge zur Kenntnis einiger Drüsen und Epithelien. *Arch. Mikrosk Anat.* 52, 552-706.
- Webber WA, Lee J. 1975. Fine structure of mammalian renal cilia. *Anat. Rec.* 182, 339-44.
- Wiederhold ML. 1976. Mechanosensory transduction in “sensory” and “motile” cilia. *Annu Rev Biophys Bioen* 5,39-62.
- Wheatley DN. 1969. Cilia in cell-cultured fibroblasts. I. On their ocorrence and relative frequencies in primary cultures and established cell lines. *J. Anat.* 105, 351-62.
- Wheatley DN. 1971. Cilia in cell-cultured fibroblasts. II. Relationship between mitotic activity and cilium frequency in mouse 3T6 fibroblasts. *J. Anat.* 110,367-82.
- Wheatley DN.1972. Cilia in cell-cultured fibroblasts. IV. Variation within the mouse 3T6 fibroblastic cell line. *J. Anat.* 113, 83-93.
- Wheatley DN. 1995. Primary cilia in normal and pathological tissues. *Pathobiolog.* 63, 222-38.
- Whitfield JF. 2008. The solitary (primary) cilium--a mechanosensory toggle switch in bone and cartilage cells. *Cell Signal.* 20(6):1019-24.
- Wilson RB, Mc Whorter CA. 1966. Isolated flagella in human skin. *Lab Invest.* 12, 242-49.

ZOOPLANKTON COMPOSITION IN AN ESTUARINE AREA OF THE STATE OF MARANHÃO, NORTHEASTERN BRAZIL

Sérgio Luiz Costa Bonecker and Cristina de Oliveira Dias*

Universidade Federal do Rio de Janeiro, Instituto de Biologia,
Departamento de Zoologia, Laboratório Integrado de Zooplâncton e Ictioplâncton,
CCS, Bloco A, Ilha do Fundão, CEP: 21941-590, Rio de Janeiro, RJ, Brazil

ABSTRACT

The Gulf of Maranhão is the largest and more important estuarine complex in the state of Maranhão, Brazil. São Marcos Bay located in the northeast region of the gulf, contains many small estuaries. The water circulation is due mainly to the tidal currents. This report describes the structure of the zooplankton community in relation to hydrographic conditions in the area, during the dry season/2000. The zooplankton samples were obtained in horizontal tows using a net with 200 μm mesh size. Temperature, salinity, dissolved oxygen and chlorophyll-*a* were measured in water samples. Temperature and salinity were homogeneous throughout the water column. The highest zooplankton densities were observed in São Marcos Bay (up to 19,500 ind.m⁻³), and the lowest were recorded in the Cachorros River and Coqueiros Strait (less than 8,000 ind.m⁻³). Fifty zooplankton taxa were identified. The copepods, decapods and chaetognaths represented 99% of the total density. *Parvocalanus crassirostris*, *Acartia tonsa*, *Subeucalanus pileatus* and *Oithona hebes* were the most abundant species. *Parasagitta tenuis* and *Ferosagitta hispida* were also abundant. Zooplankton diversity varied from 1.33 to 1.71 bits.ind⁻¹. The diversity index followed the variations in the density, and was similar to those found in other coastal systems of northeast Brazil.

Keywords: Zooplankton, Spatial variation, Estuarine area, São Marcos Bay, Brazil.

1. INTRODUCTION

The Brazilian coast has many estuarine regions of different origins and geomorphology (Bonecker et al. 2007). The Gulf of Maranhão is the largest and most important estuarine

* Autor para correspondência: Cristina de Oliveira Dias - crcldias@hotmail.com

complex in the state of Maranhão. The gulf is located between the coast of the state of Amazonas and the northeastern coast of Brazil. In its central region, the São Luís do Maranhão Island separates the gulf into two great bays: the São Marcos Bay in the west, and the São José Bay in the east (Stride 1992). São Marcos Bay is a tropical estuary, and is unusual because of the wide range of tidal amplitude (Stride 1992). Similar characteristics to those of São Marcos Bay are found in the Rance estuary in northwest France, where tidal amplitudes of 13 m occur; and also at Pico Island in the Azores (Bonecker et al. 2007).

The study site is defined by the region formed by the São Marcos Bay, the Mosquitos Strait and the Cachorros River at 02°18'-02°47' S and 044°20'-044°25' W. Since 1980, many studies have been carried out in the region of the Gulf of Maranhão, the majority of these on aspects of the biology and fishery of shrimp and fish (Castro and Emerenciano 1978, Ramos-Porto et al. 1978, Coelho and Ramos-Porto 1980, Porto 1983/84).

While numerous studies exist on the zooplankton community structure in the neritic or epipelagic zone, information about the northern Brazilian region is extremely limited. Lopes (1988), Gonçalves de Lima (1996), Silva et al. (2003; 2004), Gonçalves et al. (2004) and Krumme and Liang (2004) studied the northern and northeastern coast of Brazil. The composition and occurrence of zooplankton in the Coqueiros and the Mosquitos straits and the Cachorros River were evaluated by Paranaguá et al. (1984) and Lopes (1986).

The aim of this study is to describe the occurrence and abundance of the zooplankton community in the Gulf of Maranhão (São Marcos Bay, the Mosquitos Strait and the Cachorros River). This community is compared with others located in different coastal regions. The relationships of the zooplankton community to environmental factors are also examined. Few estuaries on the Brazilian northeastern coast have been surveyed, and this area is the subject of the fewest studies on the estuarine and coastal zooplankton communities (Brandini et al. 1997). Only abstracts, dissertations and theses exist, which increases the importance of this study.

2. MATERIAL AND METHODS

2.1. Study Area

São Marcos Bay is located on the central coast of the state of Maranhão, where the Maranhão Gulf is located (Figure 1). The bay is an estuary approximately 100 km long and up to 16 km wide. It receives several rivers, including the Grajaú, Mearim and Pindaré. Mangroves and marshes are dominant in this region (Juras et al. 1983, Camargo and Isaac 2003). The range of tidal amplitude can reach 7 m. In the bay mouth, the flood tide flows to the northwest and the ebb tide flows to the north, with a current speed of 3.9 knots (Stride 1992). The climate is typically equatorial humid, with a rainy season (November through June) and a dry season (July through October).

The mixing process of saline and continental waters, high primary and secondary production, and high concentrations of nutrients and suspended matter characterize the waters of this region as typically estuarine (Juras et al. 1983). This is the largest and most important estuarine complex in the state of Maranhão.

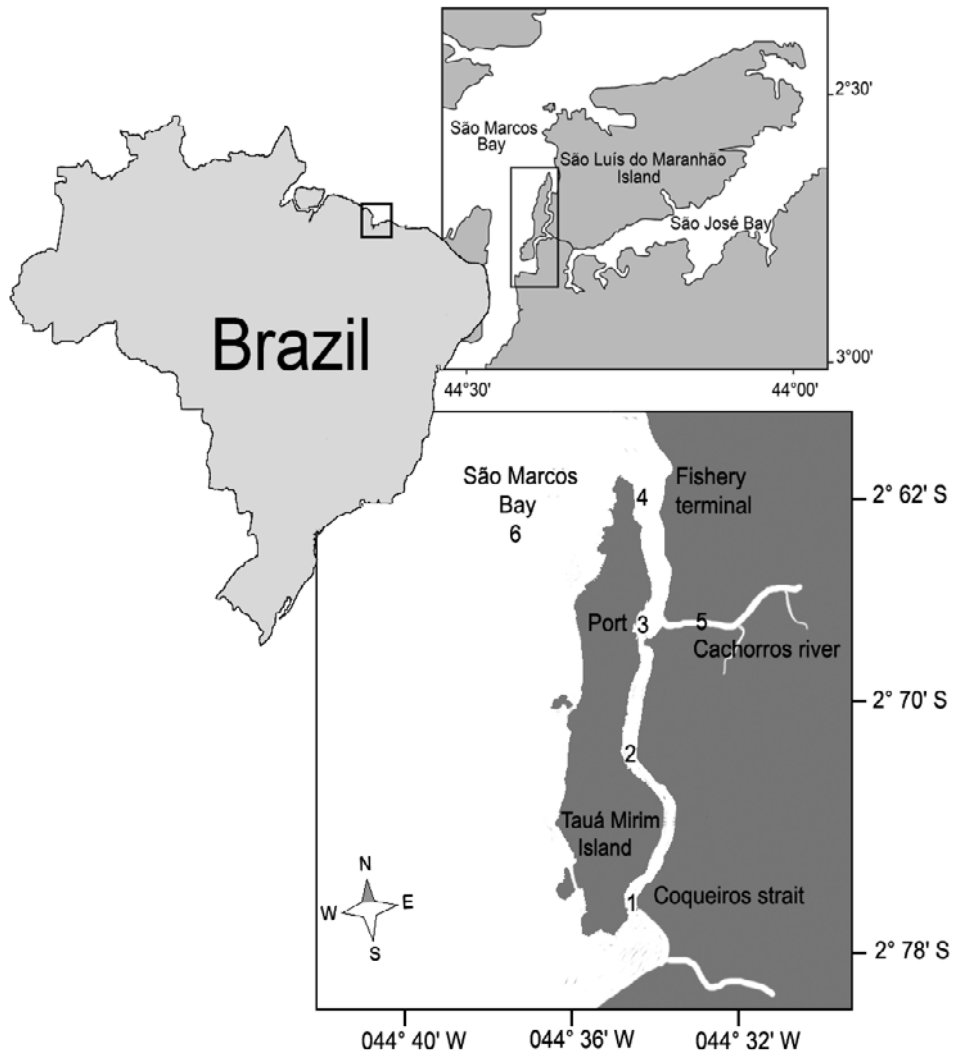


Figure 1. Locations of the sampling stations in an estuarine area in the state of Maranhão, northeastern Brazil.

South-southwest of São Luís do Maranhão Island ($02^{\circ}38'12''$ - $02^{\circ}43'14''$ S and $044^{\circ}23'35''$ - $044^{\circ}17'50''$ W) are the Coqueiros and Mosquitos straits and the Cachorros River. The Mosquitos Strait ($2^{\circ}38'12''$ S - $44^{\circ}23'35''$ W) separates São Luís Island from the continent.

This strait is 5 km long and 104 m wide, extending southeast-northwest, and linking Arraial Bay with São Marcos Bay (Nogueira and Ferreira-Nogueira 2001). The Coqueiros Strait is a north-south channel, limited to the north by São Marcos Bay and to the south by the Mosquitos Strait, which joins it to São José Bay. Draining into the northern part of the Coqueiros Strait and 3.5 km distant from São Marcos Bay, the Cachorros River is the main contributor of continental waters to the strait (Castro et al. 1989).

The hydrodynamism in the Coqueiros and the Mosquitos straits is the result of the interaction of the tidal waves proceeding from the São Marcos and São José bays. The strong

currents of the region produce intense turbulence in the water column, causing mixing and consequent resuspension of fine material (Ferreira and Kjerfve 1981).

2.2. Sampling Method and Treatment of Samples

A sampling program was carried out during the dry season (July 2000) at six stations: four in the Coqueiros Strait (stations 1 to 4), one in the Cachorros River (station 5) and one in São Marcos Bay (station 6) (Figure 1). The zooplankton samples were collected during the day at ebb tide, with the exception of station 5, which was visited at flood tide because of logistical constraints. All the hauls were horizontal at the surface, using a cylindrical-conical net of mesh size 200 μm with mouth diameter 60 cm, fitted with a calibrated flowmeter. Samples were preserved immediately after the hauls in 4% buffered formalin.

The samples were divided into fractions with a Folsom Plankton Sample Splitter (McEwen et al. 1957), and replicated subsamples containing at least 100 individuals were taken for analysis (Frontier 1981) of the more abundant groups. Appendicularia, Copepoda, Doliolidae and Chaetognatha were identified by means of appropriate references (e.g., Björnberg 1981, Bradford-Grieve et al. 1999, Casanova 1999, Esnal 1999, Esnal and Daponte 1999, Bonecker and Carvalho 2006, Avila et al. 2006 and Dias and Araujo 2006). The other zooplankton groups were counted. Species composition and abundance were determined for all samples. Shannon's diversity index (H') was calculated to evaluate the degree of organization of the copepod community (Shannon 1948).

Local depth ranged from about 10 m at stations 1 to 5, between Tauá Mirim Island and the continent, to 20 m at station 6, in the bay. Water temperature and salinity were also determined from samples taken at the surface (0.5 m), middle depth (5~10 m) and near the bottom (15~20m) using a LabComp thermosalinometer (0.1 $^{\circ}\text{C}$). Dissolved oxygen and chlorophyll-*a* were measured according to CNEXO (1983) and Parsons et al. (1984), respectively.

Analysis of variance (ANOVA) tests were used at a significance level of $p=0.05$ to identify statistical differences among the sampling stations. All data were evaluated for normality and homogeneity prior to analysis. Data were log-transformed [$\log(x+1)$] to obtain a homoscedastic data distribution (Legendre and Legendre 1983). In order to test the similarity in the abundances and compositions between stations, a cluster analysis on samples-species data matrix of the 6 stations was performed using the Euclidean Distances index.

3. RESULTS

3.1. Environmental Data

The water salinity and temperature were homogeneous ($p < 0.05$). The lowest values were observed at stations 1 and 2, between Tauá Mirim Island and the continent. At station 4, at the entrance of the Coqueiros Strait, differences between water salinity at the surface and near the bottom were greater than those found at the other stations (Table I).

During the survey period, water temperature varied more widely (6.2°C) at the surface than near the bottom. At station 4, the temperature differed more between the surface and the bottom than at the other stations. This result is similar to that for the salinity. At stations 4 and 5, colder water was found at the surface (Table I).

Dissolved oxygen content ranged between 3.6 (station 3) and 5.1 mg.L⁻¹ (station 6). The levels at the stations in the Mosquito Strait and Cachorros River were relatively lower (Table I). Chlorophyll *a* ranged between 3.4 (station 4) and 32.3 µg.L⁻¹ (station 1) (Table I). Stations 1 and 2 showed higher levels than at the other stations.

Table I. Temperature (°C), salinity, dissolved oxygen (mg.L⁻¹) and chlorophyll *a* (µg.L⁻¹) sampled in an estuarine area of the state of Maranhão, northeast coast of Brazil

Parameter	Stations					
	1	2	3	4	5	6
Temperature - surface	28.5	28.8	28.9	22.7	25.0	28.5
Temperature - mid-depth	28.5	28.5	28.5	28.5	28.3	28.5
Temperature - bottom	28.4	28.5	28.4	24.2	28.4	28.3
Salinity - surface	16.4	17.0	21.9	22.7	22.5	20.3
Salinity - mid-depth	16.5	17.2	21.9	23.8	22.4	20.9
Salinity - bottom	16.6	17.2	22	24.1	21.6	21.1
Dissolved oxygen	3.9	4.4	3.6	3.9	4.1	5.1
Chlorophyll <i>a</i>	32.3	26.7	5.9	3.4	4.3	4.9

3.2. Species Composition and Abundance

Forty taxa were identified, including 10 species of copepods and 30 species of other groups (Table II). Copepods, decapod larvae and chaetognaths represented 99% of the total zooplankton abundance. Copepods were the largest zooplankton component in the study area, in terms of both numerical abundance and species diversity. Copepods accounted for at least 94% of the total zooplankton abundance.

The average abundance of zooplankton was 8811 ind.m⁻³ (SD=8336). The highest zooplankton abundance among the six sampling stations was 19,579 ind.m⁻³ at station 6 in São Marcos Bay, while the lowest was 5393 ind.m⁻³ at station 4 in the entrance of Coqueiros Strait (Figure 2). The Cluster Analysis grouped the stations situated between the Cachorros River and the continent, which were separated from the stations located in Coqueiros Strait and São Marcos Bay (Figure 3).

The zooplankton was dominated by estuarine-coastal copepod species, *Parvocalanus crassirostris*, *Acartia tonsa*, *Subeucalanus pileatus* and *Oithona hebes*.

Table II. Minimum and maximum values, mean and standard deviation (ind.m⁻³) of the zooplankton taxa sampled in an estuarine area in the state of Maranhão, northeastern coast of Brazil

Taxa	Min.	Max.	Mean	S.D
Order Foraminifera	0.04	0.82	0.16	0.32
Class Hydroidomedusae	0.11	1.71	0.49	0.62
Phylum Nemata	0.04	0.04	0.01	0.02
Class Bivalvia (larvae)	0.04	8.02	1.77	3.13
Class Gastropoda (larvae)	0.28	0.92	0.20	0.37
Class Polychaeta (larvae)	0.2	97.71	24.61	40.21
Order Calanoida	42.36	42.36	7.06	17.29
<i>Acartia tonsa</i>	407.12	2880.59	1316.52	988.20
<i>Acartia lilljeborgi</i>	42.36	425.33	189.93	156.41
<i>Acartia</i> spp.	296.53	2456.98	975.00	848.14
<i>Parvocalanus crassirostris</i>	1186.13	8221.99	3486.11	2553.20
<i>Paracalanus</i> sp.	42.36	101.78	24.02	41.69
<i>Pseudodiaptomus acutus</i>	42.36	219.25	57.72	86.08
<i>Pseudodiaptomus richardi</i>	42.36	42.36	7.06	17.29
<i>Pseudodiaptomus</i> sp.	30.38	30.38	5.06	12.40
<i>Subeucalanus pileatus</i>	84.72	4056.18	824.09	1606.30
<i>Subeucalanus</i> sp.	211.81	1315.52	383.17	488.51
<i>Temora stylifera</i>	219.25	219.25	36.54	89.51
Family Pontellidae	50.89	50.89	8.48	20.78
<i>Pontellopsis regalis</i>	50.89	50.89	8.48	20.78
Nauplii	182.28	1315.52	573.83	560.27
Order Cyclopoida	50.89	84.72	22.60	36.61
<i>Oithona hebes</i>	60.76	931.96	434.90	285.26
<i>Oithona</i> sp.	84.72	84.72	14.12	34.59
<i>Euterpina acutifrons</i>	30.38	101.78	36.15	37.50
Class Cirripedia	0.12	3.39	0.84	1.27
Class Ostracoda	0.04	0.08	0.02	0.03
Order Decapoda	151.23	634.74	298.46	178.86
Order Amphipoda	0.04	0.1	0.04	0.04
Order Mysidacea	0.05	0.1	0.03	0.04
Order Isopoda	0.03	0.1	0.04	0.04
<i>Ferosagitta hispida</i>	0.46	88.82	22.03	34.09
<i>Parasagitta tenuis</i>	18.13	375	91.13	139.83
Chaetognatha - unidentified	0.95	25.42	8.14	11.37
Chaetognatha - juveniles	1.34	92.1	21.89	39.38
<i>Oikopleura dioica</i>	0.03	0.83	0.20	0.35
<i>Oikopleura rufescens</i>	0.12	0.24	0.06	0.10
<i>Doliolum nationalis</i>	0.03	0.1	0.02	0.04
Class Actinopterygii				
Eggs	0.03	0.05	0.04	0.02
Larvae	0.08	3.3	1.41	1.25

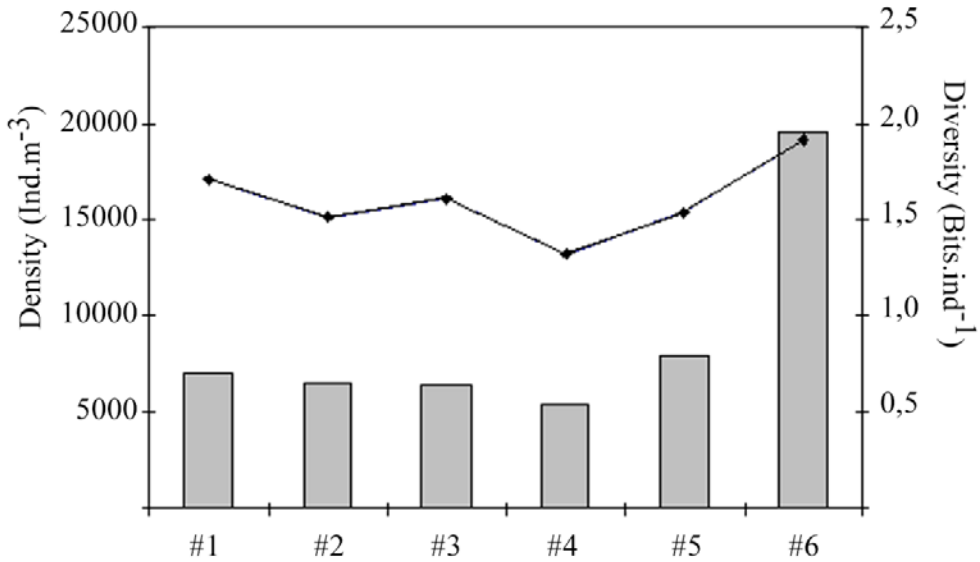


Figure 2. Total zooplankton density (Ind.m⁻³), and species diversity values in an estuarine area in the state of Maranhão, northeastern Brazil (bar= Total zooplankton density; line= Species diversity).

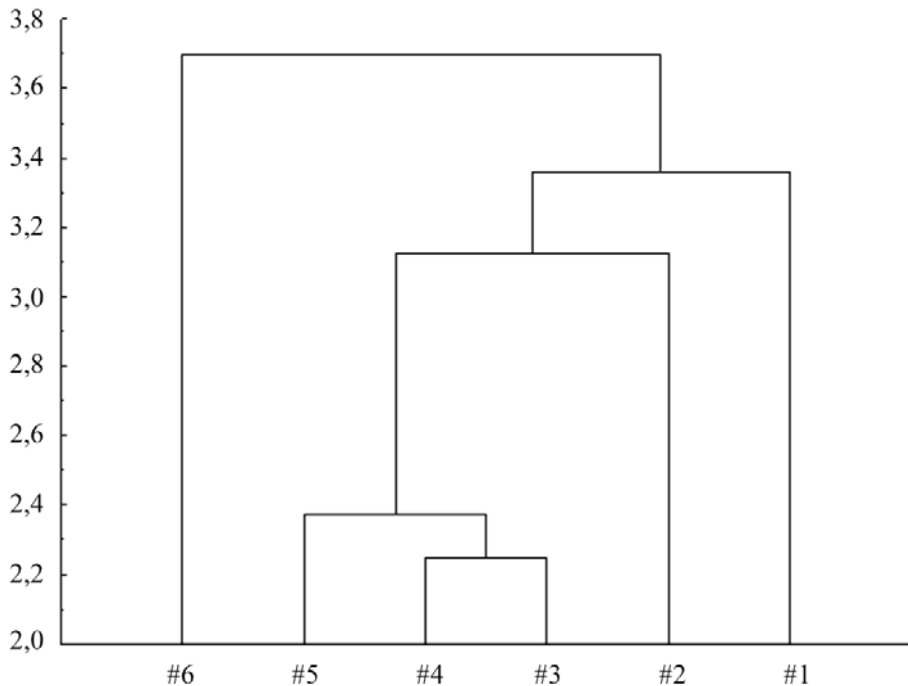


Figure 3. Cluster analysis on 15 species x 6 stations data matrix performed using the Euclidean Distances index.

Three genera of copepods (*Parvocalanus*, *Acartia* and *Subeucalanus*) dominated the zooplankton community, accounting for 81% of the total zooplankton abundance.

Parvocalanus crassirostris was the most dominant copepod in the estuarine area, constituting 40% of the total copepod abundance. It is an estuarine-marine species, and dominated in areas with salinities > 20 , with the exception of stations 1 and 2, situated between Tauá Mirim Island and the continent. The marine species *Pontellopsis regalis* was found at the entrance of the Cachorros River (station 5). An estuarine species *Pseudodiaptomus richardi* was recorded at station 2 in the Coqueiros Strait.

The Crustacea Decapoda was the second most abundant group in all stations. The highest density values (635 ind.m^{-3}) were found at station 6 in São Marcos Bay (Table II).

Other important zooplankton species in this estuarine system were *Parasagitta tenuis* and *Ferosagitta hispida* (Chaetognatha), found in all stations; and *Oikopleura dioica* and *Oikopleura rufescens* (Appendicularia), found at the inner stations 1 and 2.

In relation to the Shannon's diversity of zooplankton, station 6 (São Marcos Bay) showed the highest value (1.91). At the other stations, diversity varied from 1.33 to 1.71, with the lowest value at station 4 (Figure 2). The diversity values followed the variation of total zooplankton density values. There was practically no variation in the number of species found at each of the six sampling stations.

4. DISCUSSION

The variations of the environmental variables (salinity, temperature, dissolved oxygen) are similar to those found in the same region and in other estuarine systems (Fernandes et al. 2002, Gomes et al. 2004). The homogeneity of the water column is normal for the region, where there is no strong stratification because of the hydraulic characteristics of the bay (NUCLEBRAS 1984).

Salinities were characteristic of brackish waters. The minimum salinities were observed at the inner stations with less influence of sea water, which enters during flood tide.

The water temperature varied more in the surface samples, which were probably directly influenced by the air temperature and solar incidence. The greatest stratification of temperature and salinity occurred at station 4. This station is directly influenced by the sea water from the Coqueiros Strait, entering to the mouth of the bay, as much as by the fresh water from the rivers that discharge in this strait.

Dissolved oxygen was relatively low, characteristic of locations that receive a large organic load. The highest values of dissolved oxygen found at station 6 probably indicate the greatest influence of clearer water from the sea, because this station is in the mouth of the São Marcos bay, rather than the Coqueiros Strait or the Cachorros River.

The higher values of chlorophyll *a* can be explained by the location of the stations closer to the interior of the continent, where the input of nutrients from rivers is higher and discharge into the Coqueiros Strait, leading to a greater increase in the amount of phytoplankton, reflecting the eutrophization potential. The higher values observed at the station in São Marcos Bay may be related to its location in an open area, where greater exchange with the adjacent coastal region possibly occurs.

The zooplankton was composed by estuarine and estuarine/marine species, similar to assemblages recorded in the same region (Paranaguá et al. 1984, Lopes 1986) and in other estuarine regions in São Marcos Bay (Gonçalves et al. 2004). These authors also recorded

copepods, decapod larvae and chaetognaths as the dominant groups. In relation to the Copepoda, *Parvocalanus crassirostris*, *Acartia tonsa*, *Subeucalanus pileatus* and *Oithona hebes* were the most important. The dominant species remained nearly unchanged, which demonstrates that the region is in balance. *Parvocalanus crassirostris*, an estuarine-marine species found in brackish waters of estuaries and mangroves (Björnberg 1981), was more abundant in this area. This species is frequent in Brazilian coastal waters (Björnberg 1981), and was best represented at the station in São Marcos Bay. The marine species *Pontellopsis regalis* was found at the entrance of the Cachorros River.

The Crustacea Decapoda was the second most abundant group. The high densities of decapod larvae in the area could be explained as a consequence of the concentration of zoeae in estuarine and coastal waters. The high numbers of some larvae, juveniles and post-larvae at the entrance of the Cachorros River may be related to the collections having been made during flood tide.

This study provides the first data on species identifications of Chaetognatha, Appendicularia and Doliolidae in the region. Consequently, it is not possible to compare the results presented here with past data. Previous reports (Paranaguá et al. 1984) identified the Chaetognatha and Appendicularia to the genera *Sagitta* and *Oikopleura*, respectively. Gonçalves et al. (2004) reported the occurrence of the species *Flaccisagitta enflata* and *Oikopleura dioica* in the estuarine waters of São Marcos Bay.

The highest diversity values were found in São Marcos Bay. The values of diversity are similar to those found in other coastal systems of the northeastern Brazilian coast (Silva et al. 2003), and to those related by Margalef (1983) for coastal regions.

ACKNOWLEDGMENTS

This study was conducted in the Integrated Laboratory of Zooplankton and Ichthyoplankton, Department of Zoology, Institute of Biology, Federal University of Rio de Janeiro (UFRJ). We thank M.Sc. Paula V. Castellões for identifying specimens of the phylum Chaetognatha. We thank Janet W. Reid for improving the English text.

REFERENCES

- Avila, L.R.M.; Arruda, M.R. and Bonecker, S.L.C. (2006). Chaetognatha. In: S.L.C. Bonecker (ed.). Atlas do zooplâncton da região central da Zona Econômica Exclusiva. Rio de Janeiro, Museu Nacional, Série Livros/Documentos REVIZEE Score Central 21: 165-184.
- Björnberg, T.S.K. (1981). Copepoda. In: D. Boltovskoy (ed.). Atlas del Zooplancton del Atlantico Sudoccidental y metodos de trabajo con el zooplancton marino. *Mar del Plata*, INIDEP, 587-679.
- Bonecker, A.C.T.; Castro, M.S.; Namiki, C.A.P.; onecker, F.T. and Barros, F.B.A.G. (2007). Larval fish composition of a tropical estuary in northern Brazil (2°18'-2°47'S / 044°20'-044°25'W) during the dry season. *Pan-American Journal of Aquatic Sciences*, 2 (3): 235-241.

- Bonecker, S.L.C. and Carvalho, P.F. (2006). Appendicularia. In: S.L.C. Bonecker (ed.). Atlas do zooplâncton da região central da Zona Econômica Exclusiva. Rio de Janeiro, Museu Nacional, Série Livros/Documentos REVIZEE Score Central 21: 185-202.
- Bradford-Grieve, J.M.; Markhaseva, E.I.; Rocha, C.E.F. and Abiahy, B. (1999). Copepoda. In: D. Boltovskoy (ed.). *South Atlantic Zooplankton*. Backhuys Publishers, 869-1098.
- Brandini, F.P.; Lopes, R.M.; Gutseit, K.S.; Spach, H.L. and Sasi, R. (1997). Planctonologia na plataforma continental do Brasil - Diagnose e revisão bibliográfica. Curitiba, MMA/CIRM/FEMAR, 196p.
- Camargo, M. and Isaac, V. (2005). Reproductive biology and spatio-temporal distribution of *Stellifer rastrifer*, *Stellifer naso* and *Macrodon ancylodon* (Sciaenidae) in the Caeté estuary, northern Brazil. *Brazilian Journal of Oceanography*, 53(1/2): 13-21.
- Casanova, J.P. (1999). Chaetognatha. In: D. Boltovskoy (org). *South Atlantic Zooplankton*. Backhuys Publishers, 1353-1374.
- Castro, A.C.L. de and Emerenciano, I.A.A. (1978). Aspectos biológicos do siri-guajá *Menippe nodifrons* Stimpson, 1859, na Ilha de São Luís, estado do Maranhão, Brasil. *Boletim do Laboratório de Hidrobiologia*, 2(1): 52-76.
- Castro, A.C.L.; Martins, W.C.; Cavalcante, P.R.S. and Melo, O.T. (1989). Caracterização ambiental do estuário do Rio Paciência (MA) (I). Fortaleza, III Encontro Brasileiro de Gerenciamento Costeiro, 267-290.
- Coelho, P.A. and Ramos-Porto, M. (1980). Crustáceos Decápodos da Costa do Maranhão, Brasil. *Boletim do Instituto Oceanográfico de São Paulo*, 29(2): 135-138.
- CNEXO (1983). Manuel des Analyses Chimiques au Milieu Marin, CNEXO – BNDO Documentation (ed). Aminot, A. and Chassieupied, M., 397 p.
- Dias, C.O. and Araujo, A.V. (2006). Copepoda. In: S.L.C. Bonecker (ed.). Atlas do zooplâncton da região central da Zona Econômica Exclusiva. Rio de Janeiro, Museu Nacional, Série Livros/Documentos REVIZEE Score Central 21: 21-99.
- Esnal, G.B. (1999). Appendicularia. In: D. Boltovskoy (org). *South Atlantic Zooplankton*. Backhuys Publishers, 1375-1399.
- Esnal, G.B. and Daponte, M.C. (1999). Doliolidae. In: D. Boltovskoy (org). *South Atlantic Zooplankton*. Backhuys Publishers, 1409-1421.
- Fernandes, L.D.A.; Bonecker, S.L.C. and Valentin, J.L. (2002). Dynamic of Decapod Crustacean larvae on the entrance of Guanabara bay. *Brazilian Archives of Biology and Technology*, 45(4): 491-498.
- Ferreira, H.O. and Kjerfve, B. (1981). A pororoca no interior da Baía de São Marcos, MA. São Paulo, II Simpósio sobre Oceanografia, 138.
- Frontier, S. (1981). Cálculo del error en el recuento de organismos zooplanctónicos. In: D. Boltovskoy (ed.). *Atlas del Zooplankton del Atlántico Sudoccidental y métodos de trabajo con el zooplankton marino*. Mar del Plata, INIDEP, 163-167.
- Gomes, C.L.; Marazzo, A. and Valentin, J.L. (2004). The vertical migration behavior of two Calanoid copepods, *Acartia tonsa* Dana, 1849 and *Paracalanus parvus* (Claus, 1863) in a stratified tropical bay in a stratified tropical bay in Brazil. *Crustaceana*, 77(8): 941-954.
- Gonçalves de Lima, M.C. 1996. Hyperiids (Amphipoda: Hyperiididae) from North-Northeast coast of Brazil. *Nauplius*, 4: 147-160.
- Gonçalves, E.G.R.; Lopes, M.J.S.; de Oliveira, E.G. and Hofe, E., (2004). Associação de *Vibrio cholerae* com o zooplâncton de águas estuárias da Baía de São Marcos/São Luis – MA, Brasil. *Revista da Sociedade Brasileira de Medicina Tropical*, 37(4): 318-323.

- Juras, A.A.; Martins-Juras, I.A.B.; Castro, A.C.L. and Costa, M.L. (1983). Levantamento ictiofaunístico do estuário do rio Cururuca (município de Paço de Lumiar - Ilha de São Luís-MA). SUDEPE/UFMA. Relatório final.
- Legendre, L. and Legendre, P. (1983). *Numerical Ecology*. New York, Elsevier Scient. Publ. Company, 419 p.
- Lopes, M.J.S. (1986). Contribuição ao conhecimento da composição e ocorrência do zooplâncton na região estuarina dos estreitos dos Coqueiros e dos Mosquitos - área de influência do Consórcio ALUMAR – São Luís (MA). Salvador, II Encontro Brasileiro de Plâncton, 1.
- Lopes, M.J.S. (1988). Levantamento prévio do zooplâncton da Lagoa da Jansen, São Luiz, Maranhão (Brasil). *Boletim do Laboratório de Hidrobiología*, 8: 61-80.
- Margalef, R. (1983). *Limnologia*. Barcelona. Ediciones Omega S.A., 1010 p.
- McEwen, G.F.; Johnson, M.W. and Folsom, T.R. (1957). A statistical analysis of the performance of the Folsom plankton sample splitter, based upon test observations. *Archives for Meteorology Geophysics and Bioclimatology*, 7: 502-527.
- Nogueira, N.M.C. and Ferreira-Correia, M.M. (2001). Cyanophyceae/Cyanobacteria in red mangrove forest at Mosquitos and Coqueiros estuaries, São Luís, State of Maranhão, Brazil. *Brazilian Journal of Biology*, 61(3): 347-356.
- NUCLEBRAS 1984. Estudo do lançamento dos rejeitos da dragagem de manutenção do porto da ALUMAR na Baía de São Marcos – Maranhão. Belo Horizonte, NUCLEBRAS, Divisão de Engenharia Ambiental, DERL.PD-009/84.
- Parsons, T.R.; Takahashi, M. and Hargrave, B. (1984). *Biological oceanographic processes*. Oxford, Pergamon Press, 332 p.
- Paranaguá, M.N.; Lopes, M.J.S.; Nascimento-Vieira, D.A. and Nogueira, J.D.C. (1984). Dados preliminares sobre a composição do zooplâncton dos estuários dos rios Coqueiro, Cachorros e estreito dos Mosquitos – área de influência do Consórcio ALUMAR – São Luís (MA). Arraial do Cabo, Resumos do I Encontro Brasileiro de Plâncton.
- Porto, H.L.R. (1983/84). Determinação da estrutura da população do camarão vermelho, *Penaeus (Fanfante) penaeus* subtilis Pérez-Farfante, 1967, na ilha de São Luiz, Estado do Maranhão. *Boletim do Laboratório de Hidrobiología*, 5/6 (1): 34-53.
- Ramos-Porto, M.; Ferreira-Correia, M.M. and Souza, N.R. (1978). Levantamento da fauna aquática da ilha de São Luís (estado do Maranhão), Brasil. II Crustacea. *Boletim do Laboratório de Hidrobiología*, 2(1): 77-90.
- Shannon, C.E. (1948). A mathematical theory of communication. *Bell System Technical Journal*, 27: 379-423.
- Silva, A.P.; Neumann-Leitão, S.; Schwamborn, R.; Gusmão, L.M. de O. and Nascimento-Vieira, D.A. do. (2003). Diel and seasonal changes in the macrozooplankton community of a tropical estuary in Northeastern Brazil. *Revista Brasileira de Zoologia*, 20 (3): 439-446.
- Silva, A.P.; Neumann-Leitão, S.; Schwamborn, R.; Gusmão, L.M. de O. and Silva, T. de A. (2004). Mesozooplankton of an Impacted Bay in North Eastern Brazil. *Brazilian Archives of Biology and Technology*, 47(3): 485-493.
- Stride, R.K. (1992). *Diagnóstico da pesca artesanal marinha do estado do Maranhão*. São Luís, CORSUP/EDUFMA, 205 p.

MORPHOLOGY AND DISTRIBUTION OF FOUR PILL MILLIPEDES (*ARTHROSPHAERA*) OF THE WESTERN GHATS

B. S. Kadamannaya¹ and K. R. Sridhar^{2*}

¹Department of Zoology,
Field Marshal K.M. Cariappa Mangalore University College,
Madikeri 571 201, Karnataka, India

²Microbiology and Biotechnology, Department of Biosciences,
Mangalore University, Mangalagangothri, Mangalore 574 199, Karnataka, India

ABSTRACT

Pill millipedes, belonging to the genus *Arthrosphaera* (Family: Sphaerotheriidae) are endemic giant millipedes found mainly in the upper moist soil horizons of the forests of Southern India and Sri Lanka. They are also known as rollers as they roll into sphere or pill on conglobation as defense behavior. The bulldozing shape of body of pill millipedes helps to till and mix the soil with litter fragments, transfer organic matter into soil through burrows and uplift organic matter from underneath to generate moder humus. They hibernate into the subsoil surface on the onset of summer and attain quiescent state. Their large body size confers several advantages like tolerance to elevated moisture stress and increase the burrowing ability. The large body size is also known to affect energy acquisition and utilization, and in turn the rates of organic matter decomposition. The order, Sphaerotheriida characterized by 13 body segments (tergites) and 21 pairs of walking legs. The tergites are strongly curved and horny. Each tergite (except for pygidium) is overlapped slightly by the preceding tergite. In the process of conglobation, the head and ventral structures are completely covered by the pygidium, which helps in protection of the head and sense organs. Further, special closing ledges (locking carinae) are located on the inner side of the tergites. Stridulation (rubbing the last legs against the sides of last tergite generate sound) is an interesting phenomenon in *Arthrosphaera*. Stridulation behavior was seen usually in females and occasionally in male *Arthrosphaera* of the Western Ghats. The male stridulatory organ is the harp and in female it is washboard located at the anterior telopod and subanal plate respectively. Systematic descriptions of the order Sphaerotheriida are lacking, the current classification scheme lacks sufficient description and demands more morphological features to define monophyletic groups in *Arthrosphaera*. The current study, in addition to available morphological descriptions, emphasizes other essential features to revise the description

* Corresponding author: E-mail: sirikr@yahoo.com

of four species of *Arthrosphaera* (*Arthrosphaera dalyi*, *A. davisoni*, *A. fumosa* and *A. magna*) found in the Western Ghats along with their distribution pattern. The morphological description such as color, head, antennae, mouthparts, gnathochilarium, collum, thoracic shield, tergites, endotergum, anal shield, legs, female sexual features and male sexual features have been furnished.

Keywords: Pill millipedes, *Arthrosphaera*, morphology, distribution, Western Ghats.

INTRODUCTION

The body of millipede is generally long and cylindrical consists of three major parts head, trunk (with four simple segments) and rest of the body (with rings composed of two segments each carrying two pair of legs) (Hopkin and Read, 1992). The head capsule is heavily calcified to facilitate burrowing between soil particles, fragments of leaf litter and rotting wood. The head bears mouthparts and a number of sensory structures (antennae, Tömösvary organs and eyes). Collum, the first trunk segment behind the head is legless and each of the next three segments carries one pair of legs. Single pair of antennae possesses unique sensory cones on the last antenomere. At the base of the antennae, Tömösvary organs are present and their functions are not clearly known (Hennings, 1906; Sierwald and Bond, 2007). Median eyes are absent and the lateral patch of ocelli found in most of the millipede groups are considered as compound eye derivatives. The mouth parts consist of a pair of mandibles and the plate like gnathochilarium. According to Hennig and Mickoleit (1986) and Kaestner (1963), gnathochilarium is the fused first maxilla, while others (e.g. Kraus and Kraus, 1994) claim it to be formed by the fusion of both maxillae. The gnathochilarium consists of several sclerites that vary among orders and the homology is uncertain (Hilken and Kraus, 1994). On the ventral side of the body, structures associated with sperm transfer are present. All chilognath millipedes have a calcified cuticle (Hopkins and Read, 1992). The cuticle consists of three layers: a thin epicuticle, exocuticle and endocuticle. The cuticle of each segment consists of dorsal tergite and a ventral sternite with lateral pleurites. However, in many groups, the structure of each ring has been modified extensively. In Sphaerotheriida, tergites, pleurites and sternites are separate but in all other millipedes the pleurites are fused to the tergites to form the pleurotergal arch. Most millipedes are fairly dull in appearance or show cryptic coloration. Many species of the order Sphaerotheriida are attractively marked (e.g. red, orange and yellow spots).

The clade Pentazonia (to which pill millipedes belong) possess modified legs in males at the caudal body end (telopods) that function in sperm transfer as well as clasping the female. The structure of the gonopods is species specific and often reliable means of identification (Hopkin and Read, 1992). The pill millipedes are short compared to other millipedes with only 11-13 body segments. The Order Sphaerotheriida consists of giant pill millipedes of southern hemisphere and they possess 13 segments and roll (conglobate) into almost spheres. This Order is further organized into finer groupings including the Family Sphaerotheriidae and Zephronidae. Individuals of the order Glomerida of the northern hemisphere are also known as pill millipedes possessing 13 segments. Their second and third tergites are fused to form a very large plate. In members of both the orders the legs originate from the ventral part and consist of seven podomeres (coxa, trochanter, prefemer, femer, postfemer, tibia and

tarsus). The gonads open on or behind the second leg pair. These structures form the apertures by which sperms are introduced into the female.

The systematic treatments of the order Sphaerotheriida are scanty. However, various authors addressed this issue employing different morphological parameters for classification and same character has been named differently (Attems, 1897; Jeekel, 1974; Silvestri, 1917; Verhoeff, 1927, 1928). Recently, Wesener and Sierwald (2005a) gave a systematic scheme with comprehensive account of morphological characters used by different authors with emphasis on further standardization. In view of detailed assessment of different morphotypes of *Arthrosphaera* obtained from the Western Ghats and west coast of India in the current study, the scheme proposed by Wesener and Sierwald (2005a) has been followed. The details documented in this study hoped to fill the gaps and facilitate future studies on systematics of *Arthrosphaera*.

MATERIALS AND METHODS

Individuals of *Arthrosphaera* were collected from different locations of the Western Ghats, foothill and west coast during post-monsoon season (October-January, 2005 and 2006). The locations encompass *Arthrosphaera* species include: Kadaba (124 m); Basrikallu (1387 m); Madikeri (1147 m); Adyanadka (91 m); Adoor (110 m) and Peraje (124 m). Representative millipedes obtained were immobilized using ethyl acetate, straightened and preserved in 70% ethanol.

The dimensions of body (length, breadth and diameter on conglobation) were recorded. Morphological details of each morphotype were assessed based on observations under low and high powers of microscope.

Further details of each morphotype were evaluated on dissection and observation of various body structures after cleaning in KOH (4%): a) antennae, b) mandibles, c) gnathochilarium, d) section of endotergum, e) second leg pair in females and males, f) ninth pair of legs in females and males, g) subanal plates with washboard in females and h) anterior and posterior pairs of telopods. Detailed morphological features of each morphotype have been documented. Photographs of moving animals, conglobated animals and different segments of each morphotype have been furnished.

RESULTS

The coloration of body and different parts, specific features of head, antennae, mandibles, gnathochilarium, collum, thoracic shield, tergites, anal shield, legs, female sexual characters and male sexual characters have been given for each morphotype. The dimensions such as body length, breadth, diameter of conglobated animal (widest and narrow axis) for males and females were also provided (Tables 1-4). Distribution and ecology of each morphotype has been discussed based on the literature and the present study.

Table 1. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera dalyi* (n=10, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	7.77 (5.4-11.9)	8.94 (5.4-18.55)
Length	45.42 (39-49)	45.00 (39-50)
Breath	20.80 (16-24.0)	20.04 (15-27)
Conglobated diameter		
Widest axis	29.58 (20.2-33.8)	30.94 (27.3-34.5)
Narrow axis	23.05 (17.4-25.4)	24.05 (19.0-28.2)

Table 2. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera davisoni* (n=12, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	13.49 (8.1-16.1)	12.33 (7.3-15.1)
Length	49.83 (38.4-56.2)	45.89 (34.2-52.4)
Breadth	25.60 (18.7-31.4)	23.33 (18.4-25.7)
Conglobated diameter		
Widest axis	33.53 (26.4-38.3)	32.06 (24.3-35.7)
Narrow axis	29.26 (21.3-30.2)	26.13 (20.0-29.7)

Table 3. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera fumosa* (n=09, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	13.64 (10.80-15.1)	12.94 (9.48-15.4)
Length	48.04 (42.3-52.3)	46.27 (41.32-51.48)
Breadth	25.01 (23.6-27.0)	24.92 (23.2-27.2)
Conglobated diameter		
Widest axis	30.68 (23.8-35.0)	31.28 (26.1-35.2)
Narrow axis	26.51 (23.2-31.4)	26.16 (22-28.6)

Table 4. Weight (g) and dimensions (mm) of males and females of *Arthrosphaera magna* (n=11, mean; range in parenthesis)

Weight/Dimension	Male	Female
Weight	9.75 (7.9-13.75)	9.16 (7.8-11.75)
Length	41.18 (35-53)	39.16 (28-47)
Breadth	20.90 (16-28)	20.33 (15-26)
Conglobated diameter		
Widest axis	25.80 (19.2-33.8)	25.53 (18.4-32.43)
Narrow axis	22.69 (16-30.1)	21.93 (16.5-27.2)

***Arthrosphaera dalyi* Pocock (Figure 1)**

Color – The body is uniform deep olive brown with a reddish posterior margin of each tergite. Head and collum are dark brown, while antennae and legs are green.

Head – Polished and smooth with numerous hairs and setiferous pits around the central part and the lateral sides of the eyes. Posterior margin of the head possesses very few hairs.

Antennae – Very long with thin, long and cylindrical joints. Relative length of the antennomere: $1 > 2 > 3 > 4 = 5 < 6$, the 6th antennomere is the longest and oval tip bearing 35-40 sensorial cones. Numerous hairs are present all over the surface of each antennomere.

Mouthparts – Molar plate process of the mandible with a small furrow near the apical end with 5 pectinate lamellae with long and thin teeth and number of teeth declining proximally.

Gnathochilarium – Posterior surface with many hairs, hairs on the lingual lamella are less and a few sensorial cones are seen on the lateral of palpi.

Collum – The collum is polished, smooth with a few isolated long hairs and posterior margin with a tuft of hairs at the centre.

Thoracic shield – A few hairs are present in the concave lateral extension of the thoracic shield and marginal brim a little broaden anteriorly.

Tergites – Pubescent and punctured in front, perfectly smooth and polished in their hinder half. Anterior paratergite depression of the anterior tergites has several ridges and ridges are not seen posteriorly. Tips of the paratergite project posteriorly.

Endotergum – There is a single row of marginal bristles. Inner area is covered with a few depressions (pits).

Anal shield – It is rounded, punctured and pubescent throughout. The ventral surface carries two black locking carinae on each side. A small invagination exists at the middle of the carinae.

Legs – Tarsi of first 3 leg pair with a few ventral spines, claws only weakly curved and without an apical spine. Tarsi of the remaining pairs of legs have 6-8 ventral spines with an apical spine and curved claw. Ninth leg pair without coxal lobe. All the podomeres possess long hairs.

Female sexual characters – Second pair of legs without coxal lobe. Operculum of vulvae is long and slender, anterior margin without indentation and lower margin with indentation at the centre. Inner plate of vulvae long and broad and its anterior margin reaches over the operculum. Exterior plates are not as long as inner plate. Anterior margin of the exterior plate extends below base of the operculum. Subanal plate semicircular, hairs are present at the periphery and a dense area of hair mesially. The washboard possesses four symmetrical stridulation ribs.

Male sexual characters – *Anterior telopods*: First joint (prefemer) with a harp like structure with two stridulating ridges (one displaced to the lateral side). Femoral process situated behind the tibio-tarsus is blunt. Tibia and tarsus are distinctly separated and tibia possesses a lobe above. Tarsal process has three lateral blunt cones. *Posterior telopods*: Syncoxite with a few hairs. Coxal horns (inner horns) are dark brown and have a pointed tip. Movable finger possesses two white lobes with row of crenulated teeth. Opposite finger has crenulations juxtaposed the crenulated teeth of the movable finger.

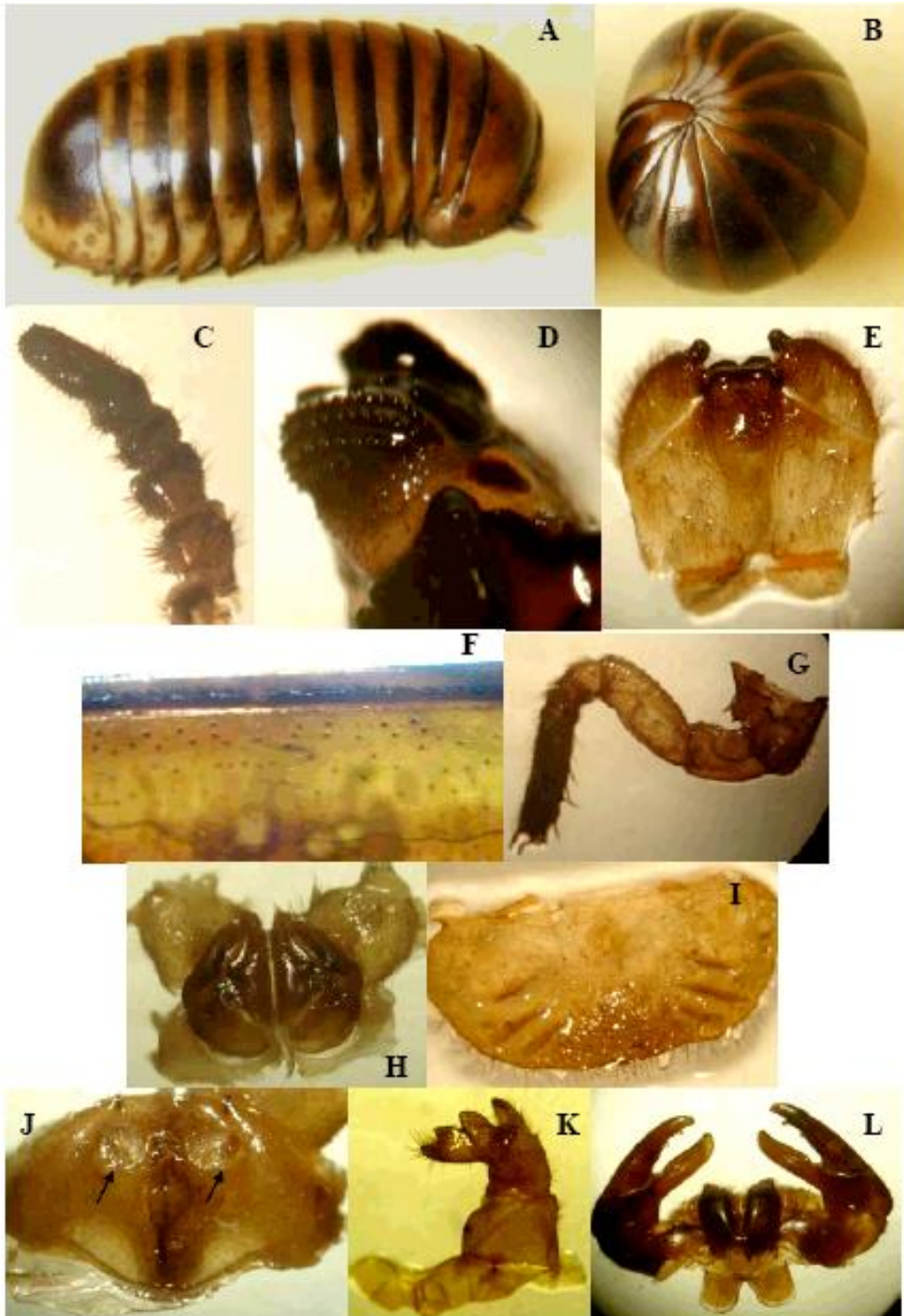


Figure 1. *Arthrosphaera dalyi* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (arrowed) (J), anterior telopod (K) and posterior telopod (L).

Distribution and ecology – The Specimens were collected from Kadaba (Western Ghat foothill location, Karnataka) at an attitude of 124 m. These individuals are abundant in

organically managed *Areca* plantation and nearby semi-evergreen forest. The animals appear immediately on onset of monsoon (end of May or early June) and could be collected in post-monsoon season up to December or January. Comparatively animals are abundant in plantation than the nearby forest. Interestingly, the number of animals could be recovered from other nearby plantation is significantly less. Probably the agricultural practices play an important role in colonization of these pill millipedes. Pocock (1995) reported this millipede from Palani Hills (1828 m) and Kodaikanal (2133 m) (Tamil Nadu). Achar (1986) has collected these animals from Alagarkovil Hills (Tamil Nadu). Chowdaiah and Kanaka (1974) have also found these animals from Southern India.

***Arthrosphaera davisoni* Pocock (Figure 2)**

Color – Body is black. Posterior portion of the tergite has a yellow band. Head is olivaceous. Collum and thoracic segment are yellowish. Antennae are olivaceous with yellow sensory area. Legs are golden brown with olivaceous tarsal segments.

Head – Possess many hairs and pits (coarsely and closely punctured). Around central clypeus and lateral sides of the eye possess hairs. The posterior margin of the head is devoid of hairs.

Antennae – Possess six joints and length of the antennomere: $1 > 2 > 3 > 4 = 5 < 6$, 6th antennomere is large and oval with more than 50 sensorial cones.

Mouthparts – Mandibles: Molar plate process with 2 steps, 7 pectinate lamellae with long teeth and number of teeth declining proximally.

Gnathochilarium – Possesses many hairs and number of hairs on the lingual lamellae is less. Lingual lamellae has 15-20 pits with hair (resembles sensory cones).

Collum – Anterior margin with some isolated hairs and posterior margin devoid of hairs.

Thoracic shield – Lateral extension of the thoracic shield has a few hairs and rim around anterior margin slightly broader than rest of the region.

Tergites – The middle of the upper surface of terga densely punctured and less densely in front and behind. The posterior tergites are pubescent in front.

Endotergum - Anterior paratergite has depression with a few hairs and ridges. Tips of the paratergite process project posteriorly. Posterior half of the terga not polished.

Anal shield – Rounded, densely punctured throughout and a pit like depression on the anterior inferior angle. Ventral surface carries two black locking carinae on each side.

Legs - First few pair of legs without coxal lobe, 9th leg has a coxal lobe with two spine-like projections and tarsi with 4-6 ventral spines and apical spine has curved claw. All the podomeres have long isolated hairs.

Female sexual characters – Second pair of leg is without coxal lobe and operculum of the vulva is long without central invagination. Lower side has an indentation, which is black. Inner plate of vulvae long and broad, and its anterior margin reach above the end of operculum. Exterior plate is not as long as inner plate and subanal plate rounded. The washboard has 5 ridges on one side and 6 stridulation ridges on the other side. Hairs are present at the periphery and also at the central portion of washboard.

Male sexual characters – Gonopores present in the second leg pair without coxal lobes. **Anterior telopods:** First joint has small harp-like process having 2 stridulatory ridges. Femoral process situated beside the tibio-tarsus, small and stout. Tibia and tarsus are

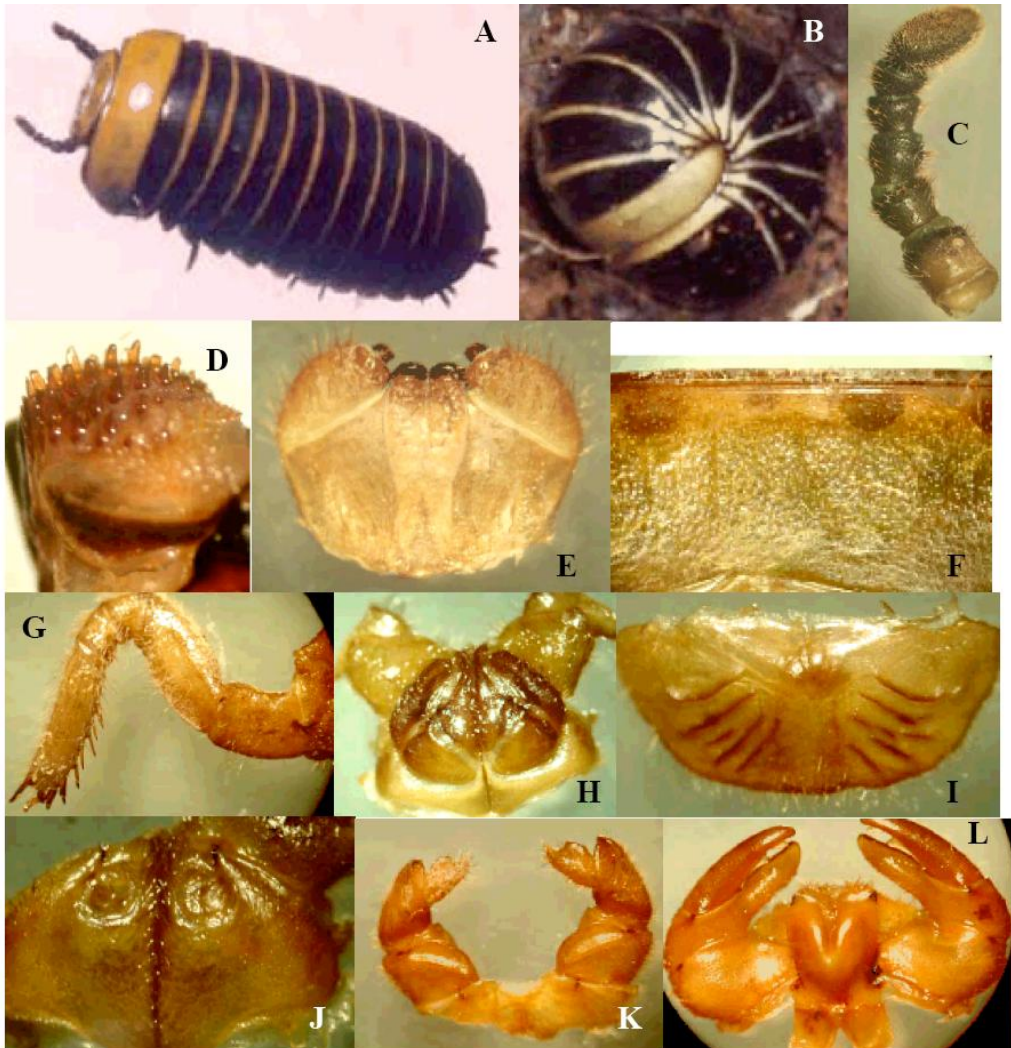


Figure 2. *Arthrosphaera davisoni* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (J), anterior telopod (K) and posterior telopod (L).

separated, former has single lateral lobe, and the latter with 2 lappets and thick spines on the inner edge. *Posterior telopods*: Coxal horns of the syncoxite of the posterior telopods with a dark lateral claw at the apex and inner horn is hairless. Movable finger has crenulations with specific dentition. Two white lappets are present on the movable finger. *Distribution and ecology* – In our study, this species was found at Basrikallu plantation, Kudremukh region (Karnataka). The plantation is situated on the sloppy landscape of the Western Ghats and consists of *Areca*, cardamom, pepper and coffee. The abundance in nearby forest is lesser than the plantations. These animals have been reported from Coimbatore, Annamalai Hills (1432 m) (Tamil Nadu) (Attems, 1936). Achar (1986) collected this species from Khandala hilly tracts of Maharashtra.

***Arthrosphaera fumosa* Pocock (Figure 3)**

Color – Pale olivaceous body, posterior half of the tergites except for the second and anal shield are smoky black, legs and antennae are deep olive to green. Nuchal plate (collum) with pale blotches and black spots.

Head – With numerous hairs and setiferous pits around the clypeus. Posterior margin of the head bears short hairs.

Antennae – It bears 6 antennomers, 6th one is the largest and broadest bearing numerous sensorial cones, the 6th joint bears terminal disc of oval or oblique shape, length of the antennomeres $1 > 2 > 3 = 5 < 6$.

Mouthparts – Mandible with seven rows of pectinate lamellae, number of teeth declining from apical to proximal. Molar plate process with two steps near apical end.

Gnathochilarium – Ventrally it has a few hairs, group of sensorial cones present on the lateral margin of palpi. Central pads bear hair and different types of sensory cells, and some with central pit and some without pits.

Collum – Anterior margin possesses a few short hairs and remaining portion is bald.

Thoracic shield – A few short hairs present in the lateral extension of the thoracic shield. Anterior marginal brim is only a little broader than rest of the brim, polished and minutely punctured.

Tergites – Anterior portion of tergites is rugose and punctured, posterior portion is smooth and highly polished. Anterior paratergite depressions have a few short hairs. Anterior paratergite depressions of the a few anterior tergites consist of ridges. Tips of the posterior margins of paratergites are slightly projecting posteriorly.

Endotergum – Endotergum has one row of marginal bristles, and internal section with short spines and a few isolated bristles and nodules.

Anal shield – Rounded, densely and closely punctured with many small hairs. Ventral side has two black locking carinae on both sides.

Legs – Tarsi of first two legs with a few ventral spines (3-4) and possess weakly curved claws. Tarsi of following leg pairs with 9-10 ventral spines, and apical spine and curved claw. Coxae of the 9th pair of legs devoid of lobe but the outer rim have many small black triangular spines. Podomeres possess many long hairs.

Female sexual characters – Subanal plate with washboard consisting of well-developed stridulation ribs. They are elongated and three ribs are on one side and four on the other side. Vulvae are large, covering more than two-third of the coxae. Operculum is very broad and long. Anterior margin is devoid of indentation. Exterior and inner plates of vulva are below the operculum and surrounding the basal margin of the operculum. Inner plates are long and broad, reaching almost the tip of operculum.

Male sexual characters – Male gonopore is located at inside margin of 2nd coxae. **Anterior telopods**: First joint with a small harp and two stridulation ridges. The 2nd joint (femoral process) is broad, tibia and tarsus coalesced, tibial part has a superior cone-shaped lappet and tarsal part (4th joint) has two flat lappets. Along the sides of all the four joints there are many hairs. **Posterior telopods**: Telopod is syncoxite nearly hairless. Femoral process

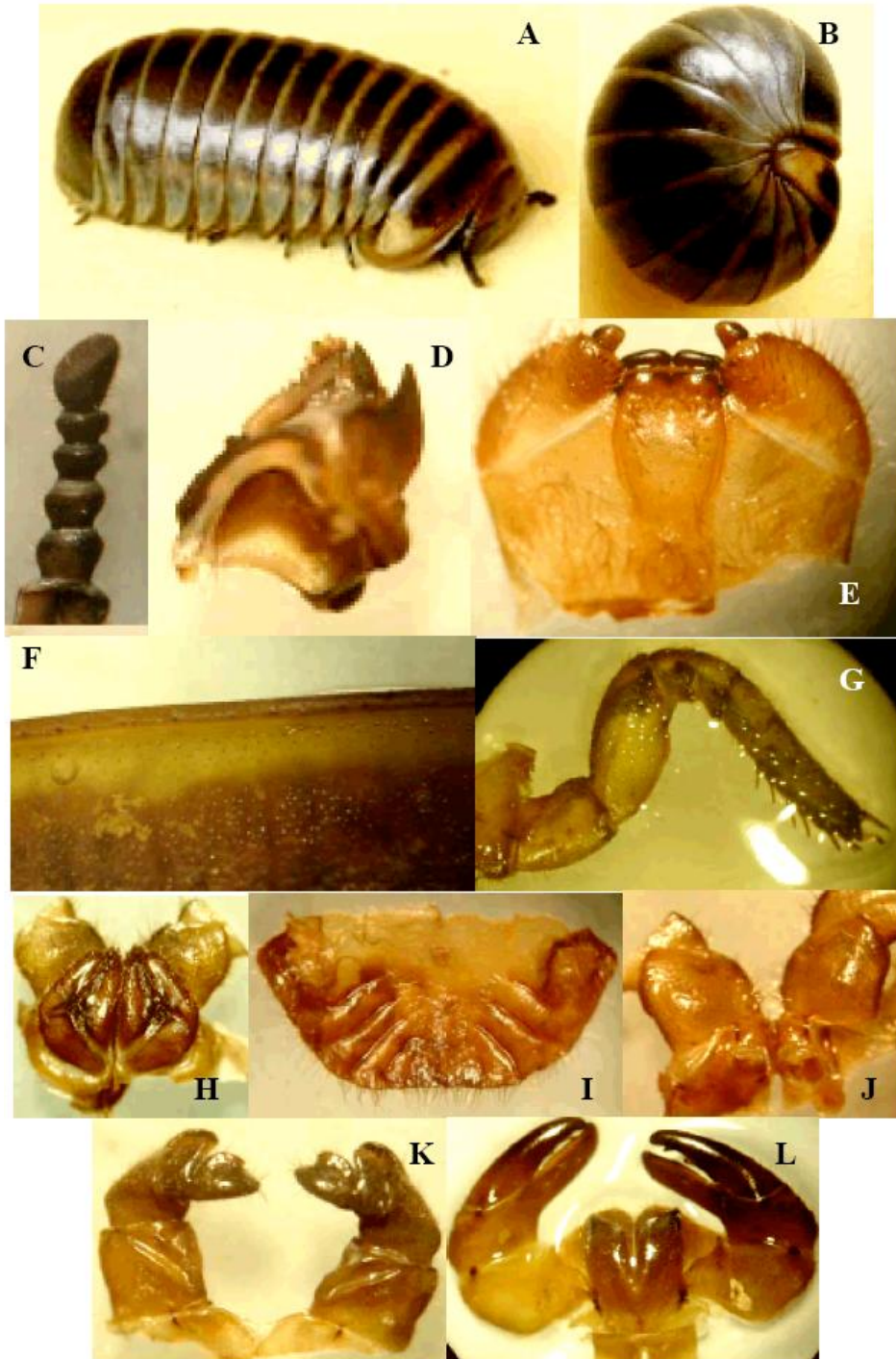


Figure 3. *Arthrosphaera fumosa* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (J), anterior telopod (K) and posterior telopod (L).

club-shaped, tibia with two white lappets and a row of numerous tubercles. The movable finger is slightly dark. The opposite finger has crenulations juxtaposed crenulated teeth of the movable finger. Inner horn of syncoxite has a pointed tip.

Distribution and ecology – Found in the semi-evergreen to evergreen forests of Madikeri and nearby locations of Karnataka. Immediately after heavy rainfall during southwest monsoon these millipedes start moving around. Interesting observation is that in addition to consuming the decomposed leaf litter, these millipedes scrape the mat of green algae and mosses attached to boulders and walls. They were recovered from Coimbatore (1432 m) (Tamil Nadu) by Pocock (1899). The animals collected resemble those collected by Achar (1986) from coffee estates of Madikeri (Karnataka).

***Arthrosphaera magna* Attems (Figure 4)**

Color – Body dark brown, posterior border of tergites yellowish forming a stripe. Head, collum and thoracic shield are yellowish brown. Antennae and legs are olive green.

Head – Head has a few setiferous pits and clypeus is not with many hairs and pits. The posterior margin of the head towards the collum has a few isolated hairs.

Antennae – Consists of 6 joints, 6th joint is expanded laterally, terminal disc is oval and oblique with more than 40 sensorial cones. The length of the antennomeres: 1>2>3>4>5>6.

Mouthparts – Mandible with 4-5 rows of pectinate lamellae, number of teeth declining proximally and molar plate process has a condyle with a single step.

Gnathochilarium – The lingual lamella with a few bristles and palpi with a few long hairs. Sensorial cones are present on the central pads and on the palpi. Sensory cells are of different type and some are with pits as well as central hairs, and the others with hairs.

Collum – Anterior margin with a row of setiferous punctures, similar row is present near the posterior border and rest of the surface is not punctate.

Thoracic shield – Polished, not punctate, anterior zone in the middle of dorsum with a few scattered punctures and each puncture possesses short hairs.

Tergites – Anterior half of the tergite is densely punctate and hairy. Posterior half is smooth and shiny with only a few punctures near the posterior margin. The anterior paratergite depressions are densely covered with hairs and tips of posterior margin of paratergite project posteriorly.

Endotergum – It shows a row of marginal bristles. The area between the marginal ridge and the internal area has many black sclerotized spots. First sternite lobe is long and reaching beyond the length of the coxa, covered with many long hairs and curved towards the leg pair.

Anal shield – Rounded entirely punctate, several punctures are united to form common shallow depressions. Anterior zone is more densely punctate with hairs. Ventral surface carries two black locking carinae on each side. They are separated by distinct suture. A small invagination is seen at the end of the suture.

Legs – Tarsi of first two legs with 3-4 ventral spines and weakly curved claw. Tarsi of next pairs of legs possess 8-11 ventral spines and a curved claw. Coxae of all legs at the inside margin densely covered with many long hairs, also on the following leg joints at the

inside margin there are some very long isolated hairs. The 9th pair of legs possesses a small tooth.

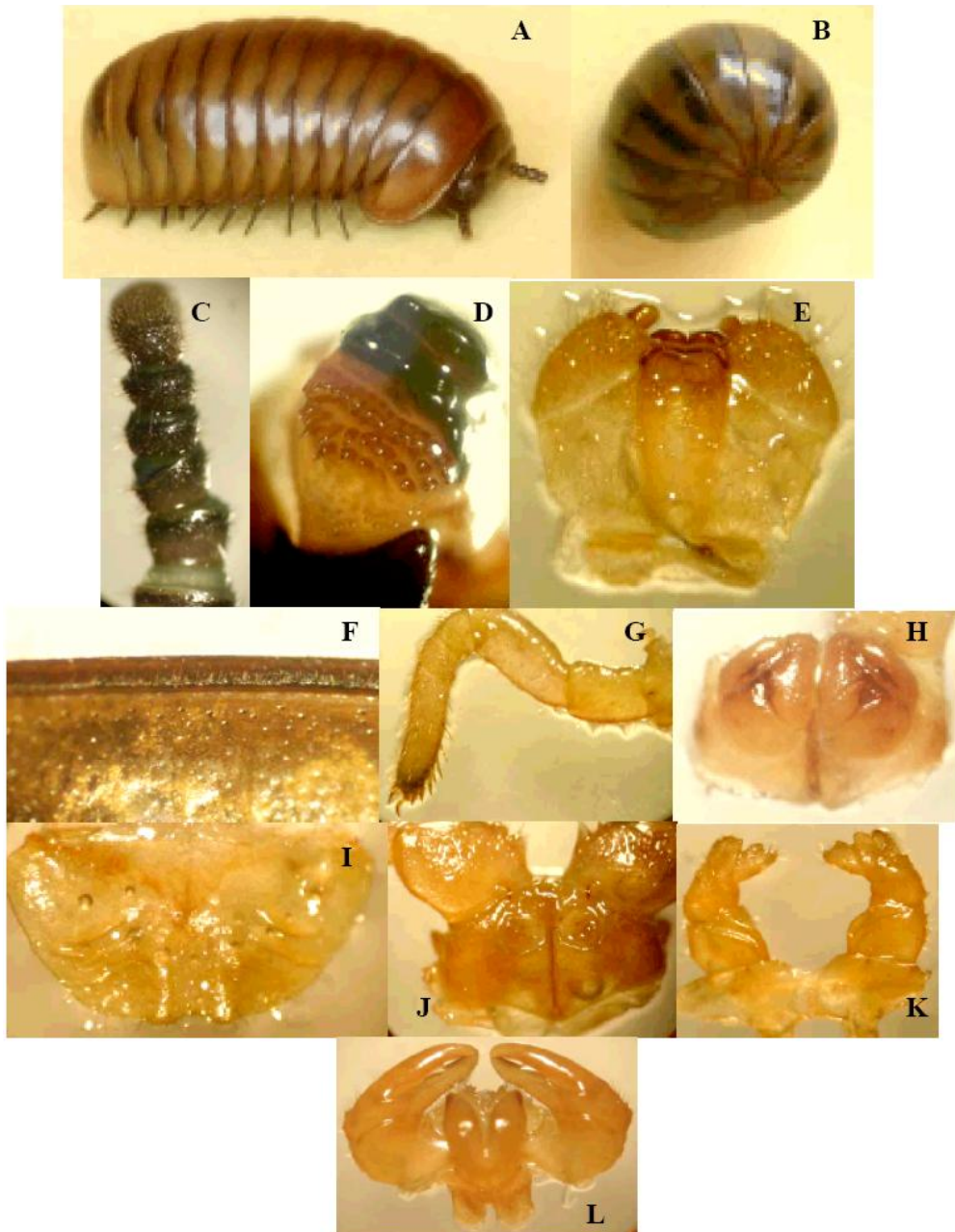


Figure 4. *Arthrosphaera magna* – Whole animal (A), conglobated (B), antenna (C), mandible (D), gnathochilarium (E), endotergum (F), 9th leg (G), vulvae (H), washboard (I), male gonopore (J), anterior telopod (K) and posterior telopod (L).

Female sexual characters – Coxae of 2nd pair of leg of female has a small lateral cone. Vulva consisting of 3 pieces, basal part is bipartite. Exterior and inner plate of the vulvae is

broad, and also the operculum is large and broad. Subanal plate is rounded and the washboard has 3 symmetrical stridulation ribs.

Male sexual characters – Second pair of leg in males is devoid of coxal lobe. Male gonopore exists on the coxae of 2nd leg pair covered with sclerotized plate. **Anterior telopods:** Possess four joints and distally synoxite. Inner margin of first joint consisting one each of long and short stridulation ridge. Femoral process (projection) is short, blunt and situated behind tibio tarsus. Tibia and tarsus (3rd and 4th joint) distinctly separated, tibia has a superior rounded lobe, tarsus with a similar lobe, and then narrowed and cylindrical with long bristles at the apex. On the posterior side it has a small knob and a small spine.

Posterior telopods: Telopod is synoxite and densely covered with hairs and coxal horns (inner horns) large, thick at the base, densely hairy on the medial side, apex is rounded and dark and thorny. Femoral process, (2nd joint) longer than tibia, crenulated teeth present on inner side. Tibia has two short broadly rounded lappets and posterior edge has numerous rounded tubercles.

Distribution and ecology – These are abundant in the forests and plantations of Western Ghats foothill. In our study, these millipedes were recovered from Adyanadka (Karnataka) mixed plantation and nearby semi-evergreen forest, and they were also found in mixed plantations of Adoor and Peraje (Karnataka). All these locations are situated in foothill of Western Ghats at an average altitude of 91-124 m. *Arthrosphaera magna* were reported from other foothill locations of the Western Ghats (Hosangadi, Mundaje, Gundya and Sampaje) (Karnataka) (Ashwini and Sridhar, 2008). Individuals of *A. magna* recovered from Mundaje and Sampaje were larger and heavier than Hosangadi and Gundya locations. Achar (1986) collected *A. magna* from *Areca* plantations of Karkala (Karnataka). There are earlier reports on collection of this millipede from Khandala Hills and north of Phenda Ghat, Kolhapur (Maharashtra), Shevaroy Hills and Woodhouse Rajamundry (Tamil Nadu).

DISCUSSION

Diplopod classification seems to be over split. More than 12,000 described species are assigned to 2947 genera in 145 families (Shelley, 2003). Among these, 68% of genera possess single or two species and 715 generic names have been considered synonyms (Sierwald and Bond, 2007). As seen in many groups of millipedes, systematic treatments are very scanty with the order Sphaerotheriida and needs specific systematic treatments. Verhoeff (1927), Attems (1897) and Silvestri (1917) gave an over view of all aspects of Diplopoda and particularly the Sphaerotheriida. Jeekel (1974) discussed the group taxonomy and geography of the Sphaerotheriida. VanderSpiegel *et al.* (2003) also attempted a revision of some of the oldest species of the genus *Sphaerotherium*. Wesener and Sierwald (2005a) revised the genus *Sphaeromimus* based on the electron microscopic study. They are of the opinion that *Sphaeromimus* shares certain characteristics with members of the Indian genus *Arthrosphaera* (e.g. four jointed anterior telopod, six jointed antennae and, flat and broad sixth antennomere), on the other hand, presence of harp on the anterior telopod relates them to the genus *Zoosphaerium*. As pointed out by Wesener and Sierwald (2005a), the morphological details of the current classification scheme are insufficient and more characters are necessary

to define precisely the species and subspecies within Sphaerotheriida. These common characters clearly indicate that the Sphaerotheriidae once inhabited the countries between Indo-Australian region and Madagascar-South Africa and subsequently they disappeared in the intermediate countries due to unfavorable climatic conditions. This is also supported by the fact that Sphaerotheriida are distributed currently in these two widely separated regions. However, the genera differ from each other in these areas with a few common characters.

Pocock (1899) listed a few characters diagnostic to the genus *Arthrosphaera* of Indian pill millipedes. These characters include: broad, truncate apex of the legs and upper angle bearing a long spine above the claw and thus a considerable space exists between the claw and the spine. In female, the vulva is composed of three distinct pieces and they are placed together in the form of a cone. In the male, the movable digit of the hinder pair of copulatory feet is composed of a single segment. These characteristics have been summarized by Attems (1936) in the key to the Indian genera of Sphaerotheriida with additional characters (coxae of the second legs of female is separated, tarsal part of the tibio-tarsus of the anterior telopods are broadly rounded lobe, margin of the lobe beset with knobs and tibia of the posterior telopods has two white lappets).

Four species of *Arthrosphaera* found in the present study are distinguishable from each other based on the characters given in Table 5. *Arthrosphaera dalyi* was reported from Alagarkovil hilly tracts of Tamil Nadu and the specimen measured 36 mm length and 29 mm width (Achar, 1986) and Palani hills (Pocock, 1895). This species collected in our study showed an average length of 45.21 mm and width of 20.42 mm. *Arthrosphaera davisoni* was collected from Basrikallu plantation near Kudremukh forest (Karnataka) in our study. The average length and breadth are 47.86 mm and 24.45 mm respectively. In the earlier studies, this species was collected from Khandla hilly tracts of Maharashtra (Achar, 1986) and Coimbatore, and Annamallai hills (Tamil Nadu) (Pocock, 1895). Pocock (1899) described this species with mean length of 31 mm for female and 22 mm for male. *Arthrosphaera fumosa* was reported from Coimbatore (Tamil Nadu) by Pocock (1895). In the present study, it was found from the forests of Madikeri and nearby forest localities of Karnataka. The average length and width of these millipedes were 47.15 mm and 24.96 mm respectively, while Pocock (1899) gave a dimension 35 mm and 16 mm. *Arthrosphaera fumosa* collected in our study resembles *Arthrosphaera* sp. (M) collected by Achar (1986) from the coffee estates of Madikeri (Karnataka). *Arthrosphaera magna* was collected from three different plantation localities at the foothill of Western Ghats. This species was reported from Karkala (Achar, 1986) and Adyanadka (Ashwini and Sridhar, 2005) of Karnataka. This species was also reported from the Western Ghats of southern India: Khandala, Phenda Ghat and Kolhapur (Maharashtra), Shevaroy hills (Tamil Nadu) and Rajamundry (Tamil Nadu). These animals were reported to measure 63 mm in length and 18.5-34 mm width (Attems, 1936), while Ashwini and Sridhar (2008) indicated as 61.57 mm length and 28.62 mm breadth. In the present study, average length and breadth are 40.17 mm and 20.61 mm respectively.

The presence of two locking carinae in some of the *Arthrosphaera* spp. reported in our study resembles the members of *Zoosphaerium* of Madagascar (Wesener and Sierwald 2005b), while one locking carina resembles the members of *Sphaeromimus* of Madagascar (Wesener and Sierwald 2005a). The subanal plates with washboard have not been described so far for the Indian species.

Table 5. Differences between four *Arthrosphaera* spp. found in the Western Ghats, India

Species	Color	Length (mm)	Width (mm)	Molar plate	Rows of teeth in pectinate lamellae	Locking carinae	Suture between carinae	Coxal lobe of legs	Stridulation ridges in the washboard	Nature of tibia and tarsus of anterior telopod
<i>Arthrosphaera dalyi</i>	Olive brown with reddish posterior margin	45.21	20.42	Absent	5	2	Present	Present	4	Separated
<i>A. davisoni</i>	Black with a yellow band posteriorly	47.86	24.46	2 steps	7	2	Present	Present	5 on one side, 6 on the other	Separated
<i>A. fumosa</i>	Pale olivaceous with smoky black posterior half	47.15	25.46	2 steps	7	2	Present	Absent	3 on one side, 4 on the other	Coalesced
<i>A. magna</i>	Dark brown with yellowish band at the posterior end	40.17	20.61	1 step	4-5	2	Present	Coxal tooth	3	Separated

Further minute details like the sensorial cones on the 6th joint of antennae, central pads of lamellae lingual, palpi of gnathochilarium and details of mandibles are lacking for *Arthrosphaera* spp. Thus, detailed study using scanning electron microscopy is warranted to throw some light on further details of Indian Sphaerotheriidae.

The stridulatory organ is considered to be absent in the *Arthrosphaera* by Attems (1936). But, in most of the descriptions of *Arthrosphaera*, the first joint of anterior telopods possesses two sharp ridges. In the present study too these ridges have been seen in all the four *Arthrosphaera* spp. Further, specimen with these stridulatory organs was treated under other genera of Sphaerotheriida. To separate tribes and subfamilies, Jeekel (1974) employed characters such as the shape of female vulva and the stridulatory organ. The shape of the bursa embracing the operculum in *Arthrosphaera* is the main synapomorphy of the genera of the family Sphaerotheriidae. Jeekel (1974) considers the presence of the female stridulation organ, the washboard, as the synapomorphy for the subfamily Arthrosphaerinae to which the genus *Arthrosphaera* belongs. The two Malagasy sphaerotherid genera, *Zoospharium* and *Sphaeromimus* from Madagascar have been classified in the Tribe Zoosphariini based on the presence of the harp in the males. The genus *Arthrosphaera* is placed under Arthrosphaeriini as it devoid of this character (Jeekel 1974). Attems (1936) in his key to Indian genera of Sphaerotheriidae clearly indicated that the characters such as basal part of the vulva bipartite and presence of two white lappets in the tibia of the posterior telopods are the important diagnostic features of *Arthrosphaera*. If the vulva is undivided and the posterior telopods or both the telopods possess stridulatory organs, they belong to other genera of Sphaerotheriidae. In the present study, pill millipedes showed the presence of ridge on the first joint of anterior telopods, vulvae are bipartite and the first joint of anterior telopod of all the species consists of two ridges (stridulatory?). The position of the ridges in anterior telopod exhibited considerable variation. So far, these ridges are not described as harp by the earlier investigators. Among the *Arthrosphaera* spp. recovered in our study, both males and females of at least a few species stridulate. The homology between the stridulatory organ (harp) and the ridge on the anterior telopods of *Arthrosphaera* need further investigation.

ACKNOWLEDGMENTS

Authors are grateful to Mangalore University for permission to carry out the study. This study constitutes part of Ph.D. thesis of BSK conducted during the tenure of Faculty Improvement Program (University Grants Commission, New Delhi, India). BSK is thankful to Dr. Pushpa Kuttanna, Principal, FMKMC Mangalore University College, Madikeri for support. We appreciate technical assistance rendered by Dr. K.S. Sreepada, Department of Applied Zoology, Mangalore University and Mr. Melwyn D'Cunha, St. Aloysius College, Mangalore.

REFERENCES

- Achar, K.P. (1986). Analysis of male meiosis in seven species of Indian pill-millipede (Diplopoda: Myriapoda). *Caryologia* 39, 89–101.

- Ashwini, K.M. and Sridhar, K.R. (2005). Seasonal abundance and activity of pill millipedes (*Arthrosphaera magna*) in mixed plantation and semi-evergreen forest of southern India. *Acta Oecologica*, 29, 27–32.
- Ashwini, K.M. and Sridhar, K.R. (2008). Distribution of pill millipedes (*Arthrosphaera*) and associated soil fauna in the Western Ghats and west coast of India. *Pedosphere*, 18, 749–757.
- Attems, C. (1897). Myriopoden. *Abhandlungen herausgegeben von der Senkenbergischen Naturforschenden Gesellschaft*, 23, 473–536.
- Attems, C. (1936). Diplopods of India. *Memoirs of the Indian Museum*, 11, 133–167.
- Chowdaiah, B.N. and Kanaka, R. (1974). Cytological studies in six species of pill-millipedes (Diplopoda-Myriapoda). *Caryologia*, 27, 55–64.
- Hennig, W. and Mickoleit, G. (1986). *Wierbellose II: Gliedertiere*. Jena: VEB Gustav Fischer Verlag.
- Hennings, C. (1906). Das Tomosvaryische organ der Myriopoden II. *Zeit. Wissen. Zool.*, 80, 576–641.
- Hilken, G. and Kraus, O. (1994). Struktur und homologie der komponenten des gnathochilarium der chilognatha (Tracheata, Diplopoda). *Verh. Nat. Ver Hambg.*, 34, 33–50.
- Hopkin, S.P. and Read, H.J. (1992). *The Biology of Millipedes*. Oxford: Oxford University Press.
- Jeekel, C.A.W. (1974). The taxonomy and geography of the Sphaerotheriida (Diplopoda). *Symposium of the Zool. Soc. London*, 32, 41–52.
- Kaestner, A. (1963). Wierbellose. In: *Lehrbuch der speziellen Zoology*, Volume 1-2. Jena: VEB Gustav Fischer, 981–1390.
- Kraus, O. and Kraus, M. (1994). Phylogenetic system of the Tracheata (Mandibulata) on Myriapoda-Insecta interrelationships, phylogenetic age and primary ecological niches. *Verh. Nat. Ver. Hambg.*, 34, 5–31.
- Pocock, R.I. (1895). Description of new genus of Zephronidae, with brief preliminary diagnoses of some new species. *Annals and Magazine of Natural History (Zoology, Botany and Geology)*, Series # 6, 16, 409–415.
- Pocock, R.I. (1899). A monograph of the pill-millipedes (Zephroniidae) inhabiting India, Cylon and Burma. *Journal of Bombay Natural History Society*, 12, 269–285.
- Shelley, R.M. (2003). A revised, annotated, family-level classification of the Diplopoda. *Arthropoda Selecta*, 11, 187–207.
- Sierwald, P. and Bond, J.E. (2007). Current status of the Myriapod Class Diplopoda (millipedes): Taxonomic diversity and phylogeny. *Ann. Rev. Entomol.*, 52, 401–420.
- Silverstri, F. (1917). Specie di Sphaeroteridae delle regioni australiana e neozelandse a me note. *Bullettina del Laboratorio di Zoologia Generale e Agraria della R. Scuola Superiore d'Agricoltura in Portici*, 12, 61–85.
- VanderSpiegel, D., Golovatch, S.I. and Hamer, M. (2003). Revision of some of the oldest species in the millipede genus *Sphaerotherium*, Brandt, 1833 (Diplopoda, Sphaerotheriida, Sphaerotheriidae), with new synonymies. *African Invertebrates*, 43, 143–181.
- Verhoeff, K.W. (1927). Myriapoda: Diplopoda. Results of Mjöberg's Swedish scientific expeditions to Australia 1910–1913. *Arkiv für Zoologi*, 16, 40–69.

- Verhoeff, K.W. (1928). Klasse Diplopoda I. In: *HG Bronn's Klassen und Ordnungen des Tierreichs* 5.2.II. Leipzig: Academy Verlag.
- Wesener, T. and Sierwald, P. (2005a). The giant pill-millipedes of Madagascar. *Proc. California Acad of Sci.*, 56, 557–599.
- Wesener, T. and Sierwald, P. (2005b). New giant pill-millipede species from the littoral forest of Madagascar (Diplopoda, Sphaerotheriida, *Zoosphaerium*). *Zootaxa*, 1097, 1–60.

COMPARISON OF NEIGHBOR-JOINING AND MAXIMUM-PARSIMONY METHODS FOR MOLECULAR PHYLOGENY OF ORYX SPECIES USING 12S rRNA AND 16S rRNA GENE SEQUENCES

***Ibrahim A. Arif, Haseeb A. Khan*, Ali H. Bahkali,
Ali A. Al Homaidan, Ahmad H. Al Farhan, Mohammad Shobrak
and Mohammad Al Sadoon***

Molecular Fingerprinting and Biodiversity Unit, Prince Sultan Research Chair for
Environment and Wildlife, College of Science,
King Saud University, Riyadh, Saudi Arabia

ABSTRACT

The molecular phylogeny using mitochondrial DNA sequences provides valuable insights for wildlife conservation. However, the inference of phylogenies may be affected by the marker type as well as by the statistical model applied for data analysis. This study reports a comparative evaluation of neighbor-joining (NJ) and maximum-parsimony (MP) methods for phylogenetic inference among three Oryx species (*Oryx leucoryx*, *Oryx dammah* and *Oryx gazella*) as well as two closely related (same subfamily but different genera) outgroups including Addax and Roan, using 12S rRNA and 16S rRNA genes. We observed that the 12S rRNA based phylogenetic inference is unable to differentiate between the genus Oryx and Addax, regardless of the method used. However, the 16S rRNA gene segment accurately grouped all the five taxa using MP but not the NJ suggesting the possible superiority of the former method for phylogenetic analysis using similar data structure. This report also describes a stepwise protocol that can easily be followed for constructing the trees using PHYLIP software.

Keywords: Mitochondrial markers; 12S rRNA; 16S rRNA; Phylogenetic trees; Oryx; PHYLIP software.

* Correspondence to: Haseeb A. Khan PhD, FRSC (UK) Chair Professor, Saudi Biological Society, College of Science, Bld 5, Room 2B42, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia, E-mail: khan_haseeb@yahoo.com

INTRODUCTION

Both 12S rRNA and 16S rRNA genes of mitochondrial DNA are commonly used for understanding the phylogenetic relationship among various taxa. The nucleotide sequences of 12S rRNA have been applied for wildlife forensic biology [1] and molecular phylogeny of endangered species [2,3]. Pandey et al [4] have utilized 12S rRNA for molecular identification of Indian leopard, which is an endangered species except in Central Africa and India. Mitochondrial 16S rRNA has been used to elucidate the pattern of relationships and systematic status of 4 genera, including 9 species of skates living in the Mediterranean and Black Seas [5]. A heminested PCR assay based on species-specific polymorphism at the mitochondrial 16S rRNA gene has been designed for the identification of seven pecora species including Blackbuck, Goral, Nilgai, Hog deer, Chital, Sambar and Thamin deer [6]. Molecular studies on endangered Pecoran have shown lower sequence diversity in 16S rRNA gene as compared to cytochrome b gene, both between and within species however the 16S rRNA gene harbored a larger number of species-specific mutation sites than cytochrome b gene, suggesting that it could be more useful for species identification [7]. NaNakorn et al [8] have assessed the level of genetic diversity of critically endangered Mekong giant catfish species using sequences of 16S rRNA and detected 4 haplotypes among 16 samples from natural populations. A single base in the 16S rDNA sequences from the endangered species *Pinna nobilis* was found to be different in all analyzed individuals from a single population sample differentiating it from the others [9]. Recently, mtDNA sequence data have been used to determine genetic diversity and phylogenetic relationship in Oryx species [10,11]. Owing to application of various molecular markers and statistical methodologies it is intriguing to compare the topologies of phylogenetic trees resulting from different computational approaches. In this investigation, we have performed a comparative evaluation of neighbor-joining (NJ) and maximum-parsimony (MP) methods for phylogenetic inference among the five antelope taxa including three Oryx species and two closely related outgroups, using 12S rRNA and 16S rRNA genes.

METHODS

We obtained the sequences of the three Oryx species including *Oryx leucoryx* (Arabian Oryx), *Oryx dammah* (Scimitar Horned Oryx) and *Oryx gazella* (Gemstok or Plains Oryx) from GenBank. The sequences of Addax (*Addax nasomaculatus*) and Roan (*Hippotragus equines*) were used as outgroups owing to their close relationship to Oryx yet representing two separate sister taxa [11,12]. The GeneBank accession numbers for 12S rRNA and 16S rRNA of the five taxa analyzed are: *Oryx leucoryx* (U86971; U87021), *Oryx dammah* (U86970; U87020), *Oryx gazella* (U86972; U87022), Addax (U86973; U87023) and Roan (U86975; U87025) respectively. There were a total of 241 and 342 positions in the partial sequence datasets of 12S rRNA and 16S rRNA respectively. The taxonomic classification of Oryx species is as follows: Kingdom (Animalia) - Phylum (Chordata) - Subphylum (Vertebrata) - Class (Mammalia) - Order (Artiodactyla) - Family (Bovidae) - Subfamily (Hippotraginae) - Genus (Oryx) - Species (*Oryx leucooryx*, *Oryx dammah*, *Oryx gazella*). The two outgroups differed at the genus level with the following classification: Genus (Addax)-

Species (*Addax nasomaculatus*) and Genus (*Hippotragus*)-Species (*Hippotragus equines*) respectively.

The sequences were aligned by ClustalW [13] and the generated alignment file was converted to PHYLIP file using BIOEDIT software [14]. The PHYLIP software was used for all phylogenetic analyses [15]. Various menus of the PHYLIP package including Seqboot (a general bootstrapping and data set translation tool; we used 1000 replicates throughout), Dnadist (a program that computes distance matrix from nucleotide sequences), Neighbor (a program that implements the neighbor-joining method), Dnapars (a program that performs unrooted parsimony) and Consense (computes consensus tree by the majority-rule consensus tree method) were used to construct phylogenies using both NJ and MP methods. The outtree file was opened with Treeview program [16] to evaluate the topologies of various tree types including radial, clad and phylogenetic trees. The entire protocol is summarized in Figure 1.

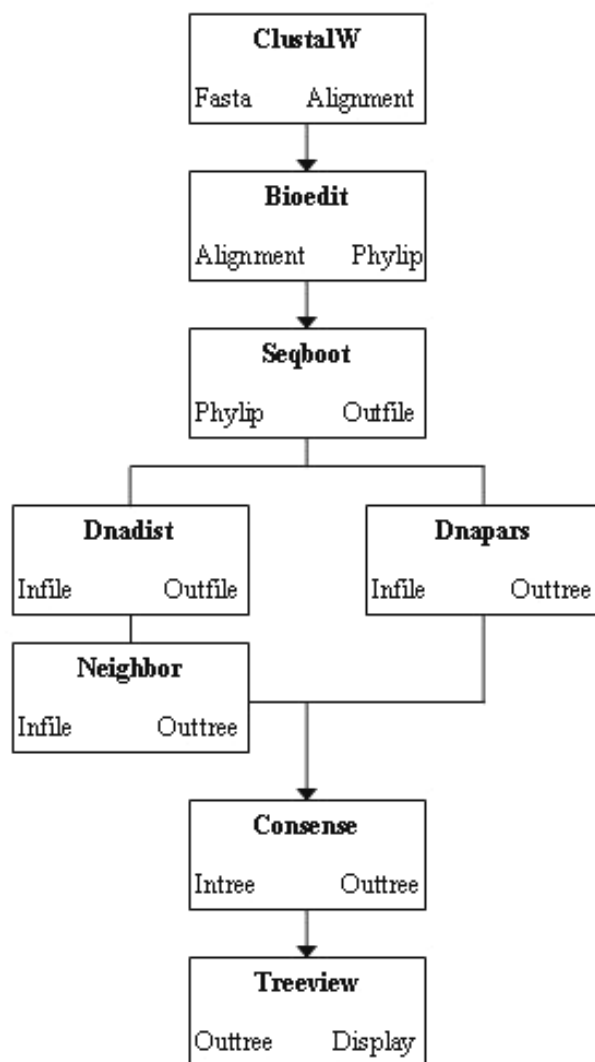


Figure 1. Schematic presentation of the protocol used. The top entry in each box indicates the software tool and the bottom entries indicate the input and the output file types.

RESULTS AND DISCUSSION

The aligned sequences of all the taxa are shown in Figure 2. The frequency of the polymorphic sites in 12S rRNA was found to be comparatively less than 16S rRNA (5.8% versus 9.1%) and so as their respective average evolutionary divergences (0.028 versus 0.043). Lei et al [17] have examined the mitochondrial rRNA genes of Chinese antelopes and observed that average sequence divergence values for 12S and 16S rRNA genes were 6.3% and 9.9% respectively. The results of phylogenetic analysis clearly showed the failure of 12S rRNA to differentiate between the genus *Oryx* and *Addax*, irrespective of the method used (Figure 3). Although mtDNA evolves faster than the nuclear genome, the rate of evolution is different for different regions of mtDNA and has been used to examine various phylogenetic relationships. Since the 12S rRNA gene is highly conserved and therefore failed to accurately differentiate taxa at genus level in our study but could be applied to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. With regard to 16S rRNA gene sequences, the NJ method did not produce satisfactory trees (Figure 4, left panel); however, the use of MP method efficiently differentiated various taxa and demonstrated the anticipated phylogenies (Figure 4, right panel). The 16S rRNA gene is regarded as a highly useful segment of mitochondrial genome for phylogenetic studies at mid-categorical levels such as families or genera [18]. The phylogenetic tree resulting from MP analysis of 16S rRNA sequences is supported by an earlier report based on using approximately 1250 base pairs long sequences of mitochondrial control region of *Oryx* species [11]. MP has been regarded as the only method that can easily tackle the insertions/deletion of nucleotides, which may provide important phylogenetic information [19]. Although MP is quite efficient in obtaining the correct topology [20], it may also result incorrect trees even if the rate of nucleotide substitution is fairly constant among the taxa [21,22].

Oryx Leucoryx						
CGGCAACGGC	CCTAAACTCA	AAGGACTTGG	CGGTGCTTTA	TACCCTTCTA	GAGGAGCCTG	60
TTCTATAATC	GATAAACCCC	GATAAACCCC	ACCAATCCTT	GCTAATGCAG	TCTATATACC	120
GCCATCTTCA	GCAAACCCTA	AAAAGGAATA	AAAGTAAGCA	TAATCATCAT	ACATAAAAAC	180
GTTAGGTCAA	GGTGTAACCT	ATGGAATGGA	AAGAAATGGG	CTACATTTTC	TACTTTAAGAA	241
Oryx Dammah						
.....G.....						60
.....						120
.....		C.		T.C		180
.....						241
Oryx Gazella						
.....		G.....				60
.....						120
.....		C.		T.C		180
.....						241
Addax						
.....				A.....		60
.....						120
.....		C.		T.C		180
.....				C.....		241
Roan						
.....	T.....		C.	C.....		60
.....		T.		A.....		120
.....			G	C.....T.		180
.....		G		A.....		241

(A) 12S rRNA

Oryx Leucoryx						
CATTTGTTCT	CTAAATAAGG	ACTTGATGA	ACGGCCACAC	GAGGGTTTTA	CTGTCTCTTA	60
CTTCCAATCA	GTGAAATTGA	CCTCCCGTG	AAGAGGCGGG	GATGAACCAA	CAAGACGAGA	120
AGACCCTATG	GAGCTTCAAC	TAACAACTC	AAAGAGAACA	AACTTAATCA	CCAAGAGATA	180
ACAGCACTCT	GTATGAGTTA	GCAGTTTGG	TTGGGGTGAC	CTCGGAGAAT	AAAAAATCCT	240
CCGAGCGATT	TTAAAGACTA	GACCCACAAG	TCAAACCAAA	TTATCGCTTA	TTGACCCAAA	300
TATTTGATCA	ACGGAATAAG	TTACCCTAGG	GATAACAGCG	CA		342
Oryx Dammah						
.....			.T.....			60
.....						120
.....						180
.T.A.....						240
.....	T..					300
.....				..		342
Oryx Gazella						
.....			.T.....	C..		60
.....						120
.....		G..				180
.....					C..	240
.....						300
.....				..		342
Addax						
.....						60
.....						120
.....	T..	..G.....	A...			180
.....	T....A..			G...	C	240
.....		T...			T....	300
C.....				..		342
Roan						
.....			.T.....	C..		60
....G....				...AG..T..		120
.....		C..	A..T..			180
...A.....	T....G....	A.....C..		C		240
.....		...TT....	T....	C.....	T....	300
.....	C....			..		342

(B) 16S rRNA

Figure 2. Alignment of 12S rRNA and 16S rRNA genes of three oryx species including the Oryx leucoryx, Oryx dammah and Oryx gazella and the two outgroups including Addax and Roan. The identical sites are represented by dots.

NJ method has been reported to give consistent trees provided the unbiased distance measures are used [23]. However, NJ method is criticized as producing only one final tree instead to generating several trees with possible correct topologies [24]. Tateno et al [25] have shown the similar efficiencies of MP and NJ methods for obtaining the correct topology of trees when the extent of sequence divergence is $\leq 5\%$ and ≥ 1000 nucleotides are used for analysis.

In conclusion, the results of this preliminary study indicate that MP appears to be a more reliable method for phylogenetic inference among the five taxa using 16S rRNA gene sequences. However, additional studies with typical sequence data sets including different marker types, sequence lengths and number of taxa are necessary to ascertain the pros and cons of MP and NJ methods in a broader perspective.

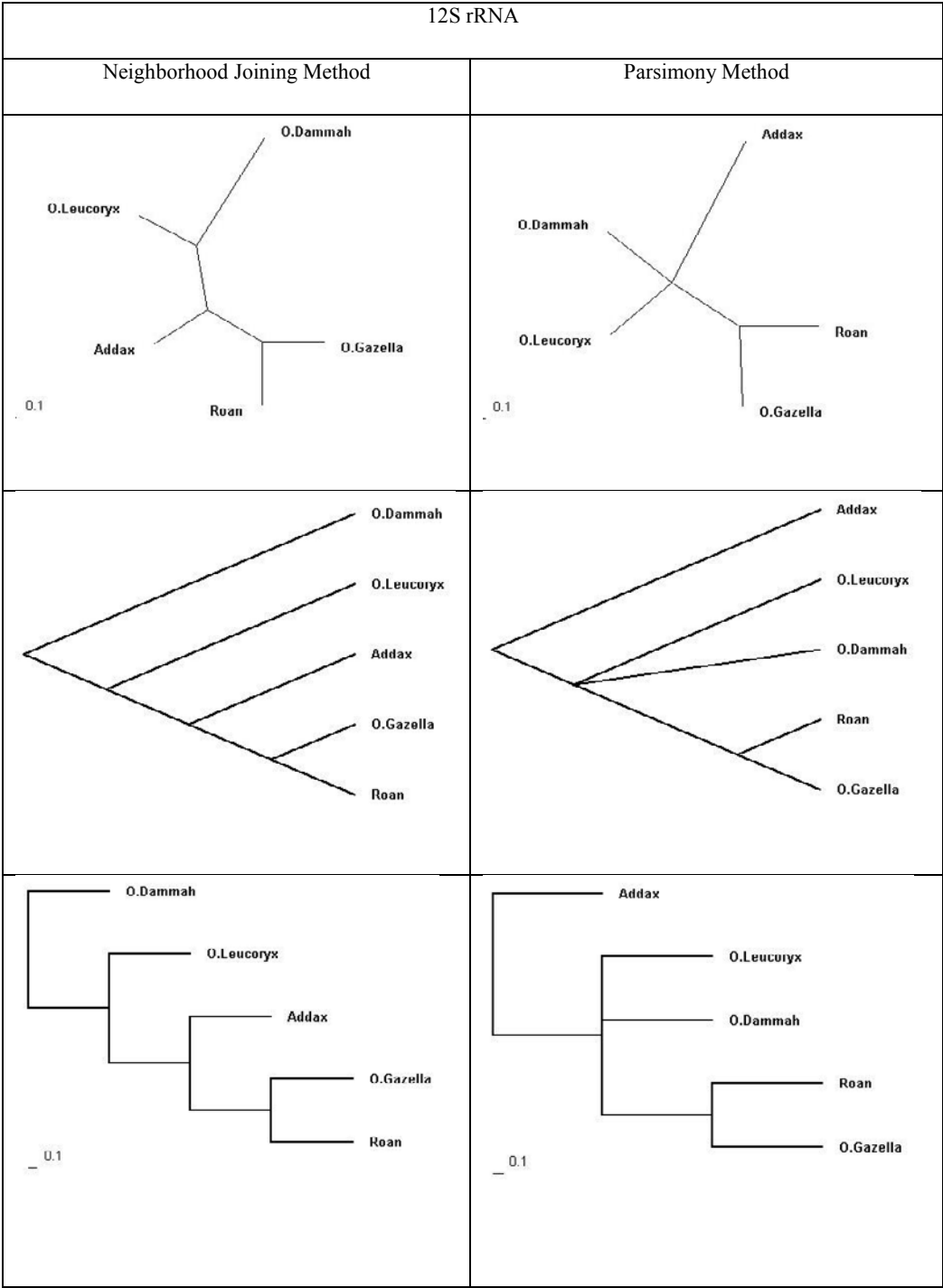


Figure 3. Bootstrap consensus trees for 12S rRNA gene using neighborhood joining (left panel) and parsimony (right panel) methods.

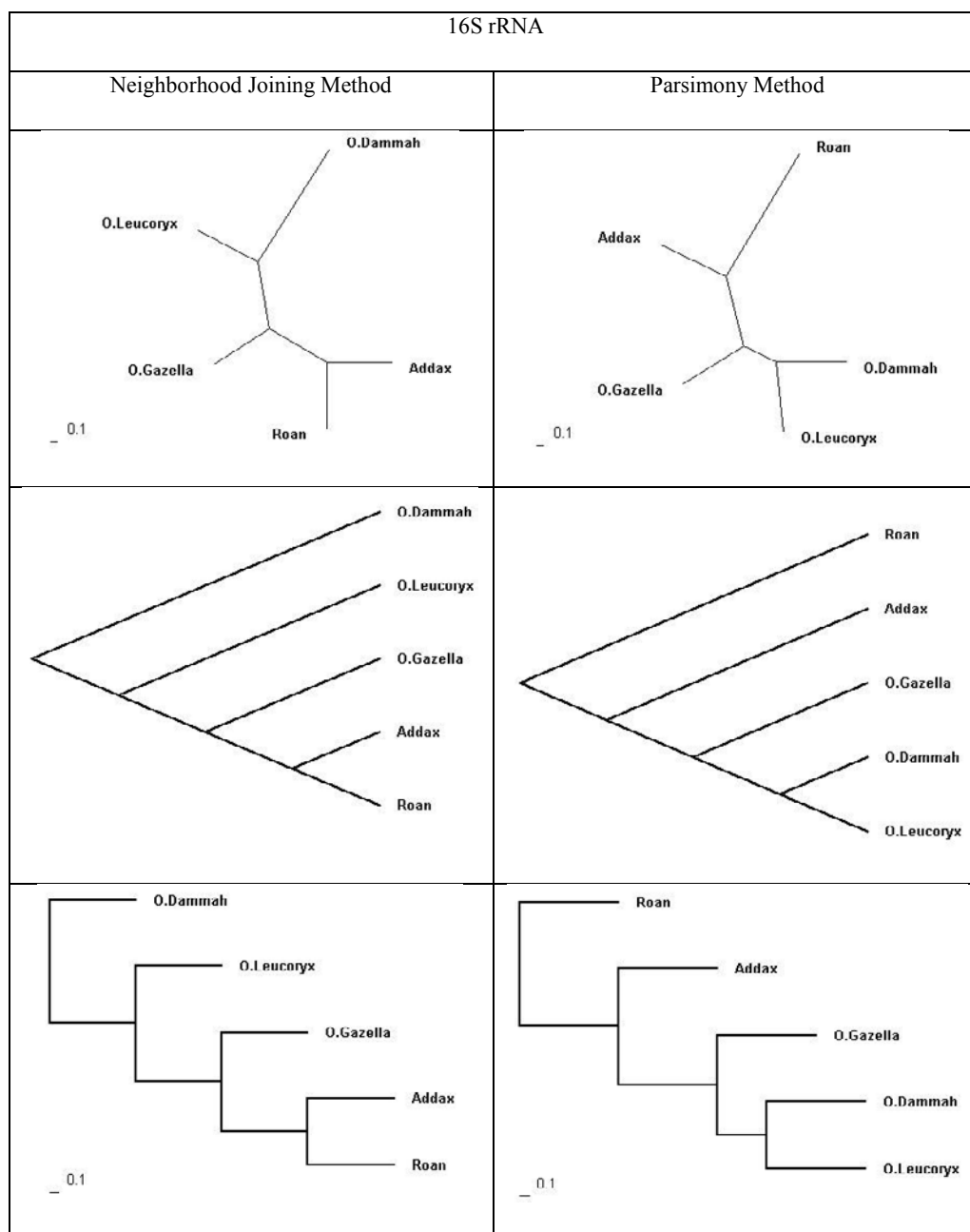


Figure 4. Bootstrap consensus trees for 16S rRNA gene using neighborhood joining (left panel) and parsimony (right panel) methods.

REFERENCES

- [1] Prakash S, Patole MS, Ghumatkar SV, Nadode SD, Shinde BM, Shouche YS (2000) Mitochondrial 12S rRNA sequence analysis in wildlife forensics. *Curr. Sci.* 78:1239-1241.

- [2] Wang CH, Kuo CH, Mok HK, Lee SC (2003) Molecular phylogeny of elopomorph fishes inferred from mitochondrial 12S ribosomal RNA sequences. *Zool Scrip* 32:231-241.
- [3] Shukla MS, Pidiyar V, Bhawe NA, Patole MS, Shouche YS (2001) PCR amplification and sequencing of mitochondrial 12S rRNA gene fragment from *Muntiacus muntjak* (Indian muntjac). *Curr. Sci.* 80:617-618.
- [4] Pandey PK, Dhotre DP, Dharne MS, et al (2007) Evaluation of mitochondrial 12S rRNA gene in the identification of *Panthera pardus fusca* (Meyer, 1794) from field-collected scat samples in the Western Ghats, Maharashtra, India. *Curr. Sci.* 92:1129-1133.
- [5] Turan C (2008) Molecular Systematic Analyses of Mediterranean Skates (Rajiformes). *Turk J. Zool.* 32:1-6.
- [6] Guha S, Kashyap VK (2005) Development of novel heminested PCR assays based on mitochondrial 16S rRNA gene for identification of seven pecora species. *BMC Genetics* 6:42.
- [7] Guha S, Goyal SP, Kashyap VK (2006) Genomic variation in the mitochondrially encoded cytochrome b (MT-CYB) and 16S rRNA (MT-RNR2) genes: characterization of eight endangered Pecoran species. *Anim. Genet* 37:262-265.
- [8] Na-Nakorn U, Sukmanomon S, Nakajima M, et al (2006) MtDNA diversity of the critically endangered Mekong giant catfish (*Pangasianodon gigas* Chevey, 1913) and closely related species: implications for conservation. *Anim. Genet.* 9:483-494.
- [9] Katsares V, Tsiora A, Galinou-Mitsoudi S, Imsiridou A (2008) Genetic structure of the endangered species *Pinna nobilis* (Mollusca: Bivalvia) inferred from mtDNA sequences. *Biologia* 63:412-417.
- [10] Masembe C, Muwanika VB, Nyakaana S, Arctander P, Siegmund HR (2006) Three genetically divergent lineages of the oryx in eastern Africa: Evidence for an ancient introgressive hybridization. *Conserv. Genet.* 7:551-562.
- [11] Iyengar A, Diniz FM, Gilbert T, Woodfine T, Knowles J, Maclean N (2006) Structure and evolution of the mitochondrial control region in oryx. *Mol. Phylogenet Evol.* 40: 305-314.
- [12] Hassanin A, Douzery EJP (1999) The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. *Mol. Phylogenet Evol.* 13:227-243.
- [13] Larkin MA, Blackshields G, Brown NP, et al (2007) ClustalW and ClustalX version 2. *Bioinformatics* 23:2947-2948.
- [14] Hall TA (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid. Sym. Ser.* 41:95-98.
- [15] Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.67. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- [16] Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Comp. Appl. Biosci.* 12:357-358.
- [17] Lei R, Jian Z, Hu Z, Yang W (2003) Phylogenetic relationships of Chinese antelopes (sub family Antilopinae) based on mitochondrial Ribosomal RNA gene sequences. *J. Zool* 261:227-237.
- [18] Gerber AS, Loggins R., Kumar S, Dowling TE (2001) Does nonneutral evolution shape observed patterns of DNA variation in animal mitochondrial genomes? *Ann. Rev. Genet* 35:539-566.

-
- [19] Nei M (1996) Phylogenetic analysis in molecular evolutionary genetics. *Annu. Rev. Genet* 30: 371-403.
 - [20] Nei M (1991) Relative efficiencies of different tree making methods for molecular data. In *Recent Advances in Phylogenetic Studies of DNA Sequences*. Oxford University Press, pp 133-47.
 - [21] Takezaki N, Nei M (1994) Inconsistency of the maximum parsimony method when the rate of nucleotide substitution is constant. *J. Mol. Evol.* 39: 210-18.
 - [22] Zharkikh A, Li WH (1993) Inconsistency of the maximum parsimony method: the case of five taxa with a molecular clock. *Syst. Biol.* 42: 113-25.
 - [23] DeBry RW (1992) The consistency of several phylogeny-inference methods under varying evolutionary rates. *Mol. Biol. Evol.* 9: 537-51.
 - [24] Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In *Molecular Systematics*. Sinauer, pp 407-15.
 - [25] Taleno Y, Takezaki N, Nei M (1994) Relative efficiencies of the maximum-likelihood, neighbor-joining, and maximum-parsimony methods when substitution rate varies with site. *Mol. Biol. Evol.* 11:261-77.

HAEMATOLOGICAL PARAMETERS IN ADULT *CHANNA PUNCTATUS* (BLOCH) SUBJECTED TO MULTISTRESS WITH AN ORGANOPHOSPHATE PESTICIDE COUPLED WITH CHALLENGE OF AEROMONADS

Rajarshi Ghosh and Sumit Homechaudhuri*

Aquatic Bioresource Research Laboratory, Department of Zoology,
University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India

ABSTRACT

An organophosphate pesticide (Methyl parathion) was presented singly and also in combination with two different strains of aeromonads, once at a time, to *Channa punctatus* (Bloch). In case of combined treatments, adult fish were exposed to a sublethal dose (0.001ml/l) of methyl parathion coupled with challenge of sublethal dose (4×10^7 cfu/ ml) of either *Aeromonas hydrophila* (MTCC 646) or *Aeromonas salmonicida* (MTCC 1522) strain injected intramuscularly. The bacterial challenge was initiated 5 days after the pesticidal exposure. The experiment comprised measurement of nine haematological parameters at 3, 7, 14, 21 and 28 days of intervals respectively. A steep increase in the total leucocytic population and corresponding leucocrit values were observed initially in combined treatments compared to fish groups exposed only with pesticides. However, all the treated groups generally showed a drop in lymphocyte population (lymphocytopenia) in the blood. Most of the erythrocytic parameters were depressed in fish groups treated only with pesticide while erythrocytic depression was less pronounced in combined treated groups.

Keywords: *Channa punctatus*, aeromonads, toxicant, stress, haematology.

INTRODUCTION

Pollution of surface waters with man-made substances, including pharmaceuticals and pesticides has been observed throughout the world and shown to cause adverse effects in

* Corresponding Author: Prof. Sumit Homechaudhuri, Department of Zoology, University college of Science, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India. Email Address: sumithomec@yahoo.com; Telephone: +91-9830392284

aquatic organisms (Bucher and Hofer, 1993; Dunier and Siwicki, 1993; Burkhardt-Holm *et al.*, 1997; Bols *et al.*, 2001). Methyl parathion (trade name: Metacid 50) is one of the most widely used organophosphate pesticide having harmful residual, transformational and bioaccumulatory properties and thus creating threat to the fish populations of ponds, lakes, muddy swamps neighboring the farmer's land. Pesticides in the aquatic environment can perturb the fish immune system and consequently, increase their susceptibility to various other pathogenic agents.

As in higher vertebrates, the blood composition of a fish by and large reflects metabolic and other physiological processes. Accordingly, the blood chemistry and haematology can be used as clinical tools for the investigation of biochemical and physiological disturbances in fish caused by pollution of the aquatic environment. Many studies have demonstrated the usefulness of haematology in the assessment of fish health (Post, 1966; Mulcahy, 1969; Cardwell and Smith, 1971; Foda, 1973; Racicot *et al.*, 1975; Shieh, 1978; Harbell *et al.*, 1979; Coles, 1986; Evenberg *et al.*, 1988; Tatner, 1990; Møyner, 1993; Řehulka, 2003; Řehulka and Minařík, 2007), and as a biomarker of exposure to pollution (Handy and Depledge, 1999).

The objective of the present experiment was to investigate haematological changes in adult spotted murrel, *Channa punctatus* (Family: Channidae) on exposure to an environmental toxicant, Metacid 50 alone and in combination with aeromonad infection (*A. salmonicida* and *A. hydrophila* respectively) with a view to assess the stress response as fish health indicator.

MATERIALS AND METHODS

A. Fish Stock Acclimatization and Maintenance

Three hundred and fifty adult *C. punctatus* of length 18.13 ± 0.31 cm and weight 83.0 ± 1.22 g (mean $\pm$ S.E.) were obtained from local fish farm (Latitude: $22^\circ 31.115'$ N; Longitude: $88^\circ 24.086'$ E) located at Kolkata, West Bengal, India. The freshly caught and healthy fish specimens of both sexes were transported live and acclimatized in the laboratory for 10 days in 60 glass aquaria (size: 0.6m x 0.3m x 0.3m, 5-6 fish per aquarium) under controlled laboratory conditions with continuous aeration. Fish were fed *ad libitum* with *Tubifex* sp. and larvae of *Culex* sp. during acclimatization period only. The water was renewed everyday to avoid accumulation of unutilized food or metabolic waste products.

B. Water Monitoring

Different water parameters like temperature, pH, hardness, dissolved oxygen and total alkalinity were measured following the standard methods (APHA, 2005). The respective values obtained are as follows: water temperature: $24 \pm 2^\circ\text{C}$; pH: 7.2 ± 0.05 ; Alkalinity(mg/l): 67.03 ± 1.56 ; Dissolved oxygen (mg/l): 3.93 ± 0.11 and Total hardness (mg/l): 240 ± 20 . The water parameters were checked before the experimental setup.

C. Toxicant

Adult *C. punctatus* were exposed to a wide range of concentrations of Metacid 50, an organophosphate pesticide before starting the present experiment. It was found that 0.00005 ml/l concentration of pesticide killed no organisms and 0.1 ml/l concentration killed all adults. For short-term definitive test, a geometrically spaced series of concentrations between 0.0001 ml/l to 0.1 ml/l was selected. To determine LC_{50} value, a 72h test with a minimum of five toxicant concentrations and a control was performed separately. The 72h LC_{50} value was recorded as 0.0125 ml/l dose with adult *C. punctatus* on static bioassay. For the present experiment, the concentration of 0.001 ml/l of pesticide was selected as a sublethal dose.

D. Bacterial Culture Collection, Maintenance and Artificial Inoculation on *C. Punctatus*

Microbial cultures of two bacterial strains viz. *Aeromonas salmonicida* (MTCC 1522) and *Aeromonas hydrophila* (MTCC 646) were collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, IMTECH, Chandigarh, India. The strains were received as lyophilized cultures and subsequently revived by adding Nutrient Broth (NB) and transferring the rehydrated culture to a fresh Nutrient Agar (NA) medium. Consequently, streak plate method was followed to get isolated bacterial colonies on a large part of the agar surface.

For determining LD_{50} (72 h) values, both strains of aeromonads (*A. salmonicida* and *A. hydrophila*) were cultured in nutrient broth (NB) and incubated at 25°C and 37°C respectively 24h prior to infectivity testing. Bacterial cells were harvested by centrifugation at 5000 x g for 5 min and washed in physiological saline, PS (0.85 % NaCl). The strains were enumerated by correlating the OD values (600nm) of the growing culture with the corresponding colony forming units (cfu) obtained by spread plate dilution method ($OD_{600nm} 1 = 2 \times 10^9$ cfu / ml). Different concentrations of bacteria ranging from 1×10^5 to 1×10^{10} cfu /ml were made up in PS (working volume: 0.5 ml /100g body weight of fish) and injected intramuscularly (im) into different groups of *C. punctatus*, each containing 10 fish. The fish were observed for changes in their behavioral patterns as well as development of haemorrhagic ulcers and tissue necrosis. The viability of the infected fish was checked for 72 h and the corresponding LD_{50} doses were determined (Reed and Muench, 1938). LD_{50} (72 h) for *A. salmonicida* and that of *A. hydrophila* were found to be 6.5×10^{11} cfu /ml and 8.0×10^{10} cfu /ml respectively. For this experiment, a sublethal concentration (4×10^7 cfu /ml) of each strain of bacteria was selected for separate intramuscular (im) injection to the pesticide-exposed adult *C. punctatus*.

E. Experimental Design

The present experiment consisted of six groups of fish in total after sufficient acclimatization under controlled laboratory conditions: three control groups (C: I-III) and three treated groups (T: I-III). Those six groups were divided into three pairs, each consisting

of one control and one treated group of fish. The detailed plan of the experiment has been illustrated in Table 1.

Table 1. Experimental design. Six groups of fish were divided into 3 pairs (I -III). CI: control; TI: pesticide exposed only; CII: sham injected control; TII: Pesticide + *A. salmonicida* (MTCC 1522) exposed fish; CIII: sham injected control and TIII: Pesticide + *A. hydrophila* (MTCC 646) exposed fish. In every group, 5 replicates of fishes were exposed for 3, 7, 14, 21 and 28 days respectively

FISH GROUPS	NUMBER OF FISH PER GROUP	FEATURES
C I	5 replicates, 10 fish per replicate	Fish kept in clean water (Control).
T I	5 replicates, 10 fish per replicate	Fish exposed to chosen sublethal dose (0.001 ml/l) of Metacid 50 (an organophosphate pesticide) alone.
C II	5 replicates, 10 fish per replicate	Sham injected and kept in clean water (Sham injected Control).
T II	5 replicates, 10 fish per replicate	Fish exposed to sublethal dose (0.001 ml/l) of pesticide and also challenged with sublethal dose (4×10^7 cfu / ml) of <i>Aeromonas salmonicida</i> (MTCC 1522). Bacterial challenge was initiated 5 days after the pesticidal exposure.
C III	5 replicates, 10 fish per replicate	Sham injected and kept in clean water (Sham injected Control).
T III	5 replicates, 10 fish per replicate	Fish exposed to sublethal dose (0.001 ml/l) of pesticide and also challenged with sublethal dose (4×10^7 cfu / ml) of <i>Aeromonas hydrophila</i> (MTCC 646). Bacterial challenge was initiated 5 days after the pesticidal exposure.

In T-I group, Fish were exposed to sublethal concentration of pesticide alone for 3, 7, 14, 21 and 28 days respectively whereas in the T-II and T-III groups, fish were exposed to sublethal doses of pesticide and also challenged with *A. salmonicida* and *A. hydrophila* respectively for the same durations as in T-I group. In both T-II and T-III groups, bacterial challenge was initiated 5 days after the pesticidal exposures. C-I control groups were present in the clean water. Moreover, C-II and C-III control groups of fish were sham injected with sterile nutrient broth (NB) where pathogens (aeromonads) were not present. During each of the five exposure period, 8 fish samples were collected from each replicate of the three control groups (C: I-III) and nine blood parameters were measured from each sample. The blood parameters studied were Leucocrit (Lct), Total Leucocyte Count (TLC), Differential count of leucocytes (DWBC %), Total Erythrocyte Count (TEC), Haemoglobin (Hb), Haematocrit (Ht) and absolute corpuscular values viz. Mean Cell Volume (MCV), Mean Cell

Haemoglobin (MCH) and Mean Cell Haemoglobin Concentration (MCHC) respectively. Simultaneously, a total of 8 samples were also collected from each replicate (1-5) of the three treated groups (T: I-III) after 3, 7, 14, 21 and 28 days of exposure respectively unless prevented by mortality. The same blood parameters were measured from each replicate of fish population.

The changes in blood parameters in these asymptomatic carrier fishes in T- I, T- II and T- III groups from their respective controls over the experimental periods were graphically represented in Figures 1-5.

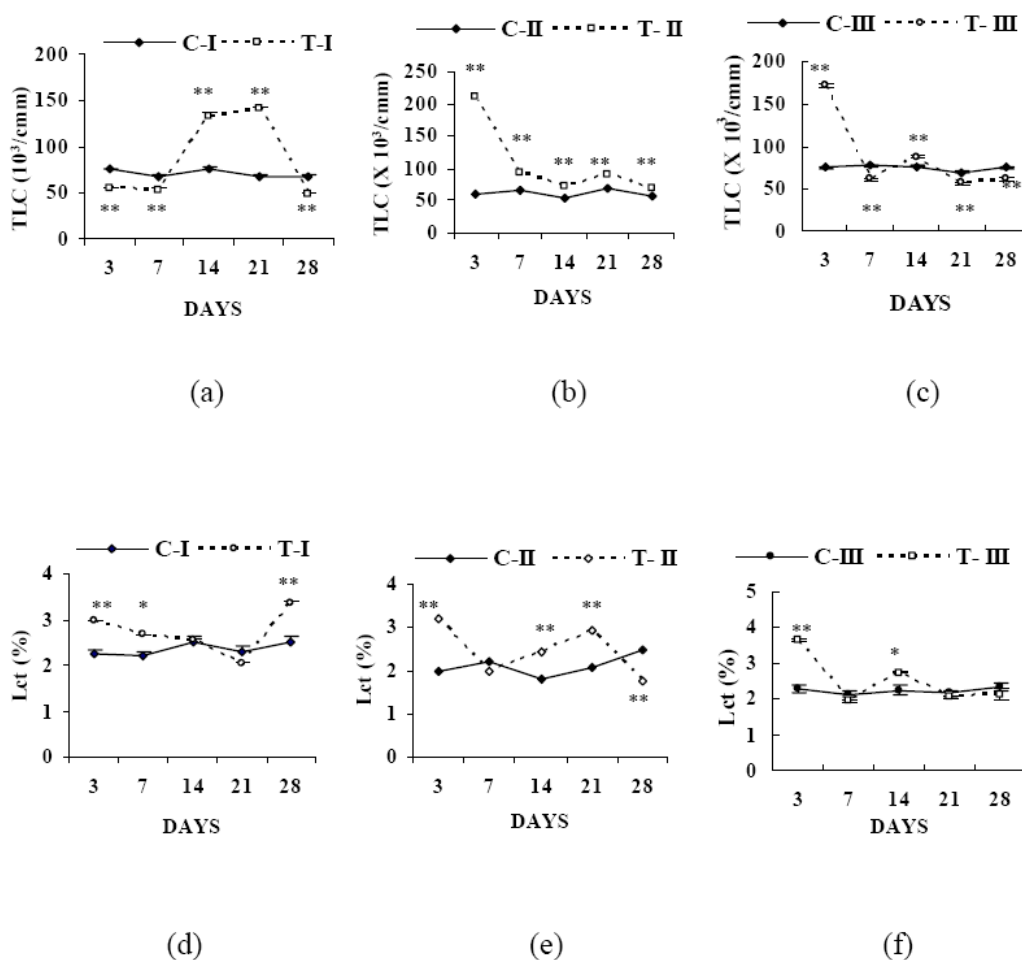


Figure 1. Changes in leucocytic parameters like TLC (a, b, c) and Leucocrit (d, e, f) respectively over time of six fish groups which were divided into 3 pairs (I -III); CI: control, TI: pesticide exposed only, CII: sham injected control, TII: Pesticide + *A. salmonicida* (MTCC 1522) exposed fish, CIII: sham injected control and TIII: Pesticide + *A. hydrophila* (MTCC 646) exposed fish; Values shown are mean $\pm$ S.E., 8 fishes for each group. * Significant ($P < 0.05$), ** Highly significant ($P < 0.01$).

F. Haematological determinations

Free flowing blood was collected by severing the caudal peduncle without using anaesthesia. Blood smears were prepared from blood free from any anticoagulant. However,

blood was collected in glass vial rinsed with 3.8 % sodium citrate (anticoagulant) for determination of Haematocrit (Ht) and Leucocrit (Lct) values.

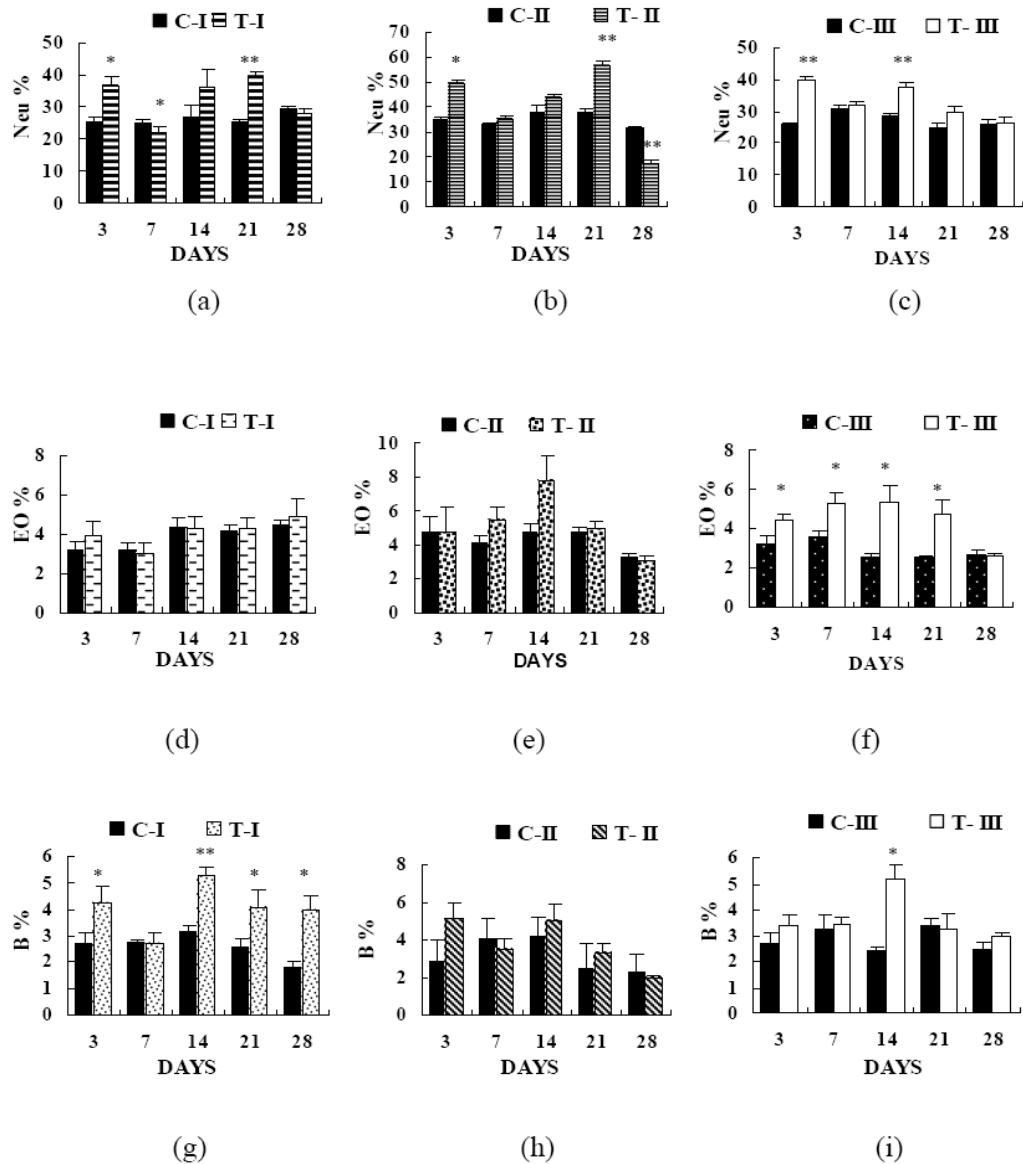


Figure 2. Changes in relative population of granulocytes (DWBC %) viz. neutrophil (a, b, c); eosinophil, (d, e, f) and basophil (g, h, i) respectively over time of six fish groups which were divided into 3 pairs (I-III); CI: control, TI: pesticide exposed only, CII: sham injected control, TII: Pesticide + *A. salmonicida* (MTCC 1522) exposed fish, CIII: sham injected control and TIII: Pesticide + *A. hydrophila* (MTCC 646) exposed fish; Means $\pm$ S.E.; 8 fish for each group; Neu: Neutrophil, EO: Eosinophil and B: Basophil. * Significant ($P < 0.05$), ** Highly significant ($P < 0.01$).

The measurements were made within 30 min of sampling. Different blood parameters viz. TLC, Lct, TEC, Hb and Ht were determined following standard methods with some modifications (Hesser, 1960; Blaxhall and Daisley, 1973; Dacie and Lewis, 1984). TLC and

TEC were made by standard clinical method (with the help of improved Neubaur double Haemocytometer, Fein-OPTIK, Blankenburg, G.D.R.) using Hayem's solution as diluting fluid. The haemoglobin content (g %) of blood was determined colorimetrically by Cyanmethaemoglobin method. The Ht and Lct values were estimated by microhaematocrit method (Wintrobe, 1967) using microhaematocrit capillary tubes and a microhaematocrit centrifuge (3000 g for 25 min). For Differential count of WBC (DWBC %), the air-dried blood films were stained with freshly prepared Leishman stain and examined under Zeiss-Axiostar plus compound microscope. The percentage of leucocytes was calculated by counting 150 cells of uniform blood smears avoiding extreme edges. The values of MCV, MCH and MCHC were calculated from the red cell count (TEC), haemoglobin (Hb) and haematocrit (Ht) values using standard formulae.

G. Statistical Analysis

The results were presented as mean and standard error (mean $\pm$ SE). Independent sample t-test was used to distinguish between means of significant differences. Treatments were taken to be significantly different where $P < 0.05$ and highly significant where $P < 0.01$.

RESULTS

A. Changes in Leucocytic Parameters

The leucocytic population (TLC) was significantly reduced ($P < 0.01$) up to 7 days of pesticidal exposure followed by a steep increase ($P < 0.01$) in the next couple of weeks in T-I group (Figure 1a). After 28 days, the population was significantly decreased again. A peak was observed after 3 days as a result of combined exposures both in T-II and T-III groups followed by a decrease in TLC (Figure 1b and Figure 1c). Overall increased values were observed in T-II group whereas highly increased TLC values were noticed after 3 and 14 days respectively in T-III group.

A steep increase in the leucocrit values (Lct) was observed after 3 days in all treated groups (T I-III) as an initial response to exposure of stressing agents (Figure 1 d-f). These findings were further confirmed by observing differential count of granulocytes (DWBC %), which showed a significant increase ($P < 0.01$) of neutrophil population in all treated groups (Figure 2 a-c). There was a significant increase in leucocrit percentage in T-I group at the termination of exposure period. This parameter also showed similar changing pattern with corresponding TLC in T-II and T-III treated groups. Neutrophil count was increased (Neutrophilia) in the middle stage while percentages of eosinophils and basophils remained high over the experimental periods in T-III (Figure 2f) and T-I groups (Figure 2g) respectively.

In spite of few significant increased populations of large and small lymphocytes observed, treatments with different stressing agents (pesticide alone or coupled with aeromonads) evoked a drop in lymphocyte population (lymphocytopenia) in the blood (Figure 3a-c and Figure 3d-f). Insignificant variations were observed in the numbers of monocytes in

T-I group. Monocyte count, however, remained low ($P<0.05$) after 7 days of exposure in T-II group of fish (Figure 3h) whereas higher percentage was obtained after 3 days of exposure in T-III group (Figure 3i).

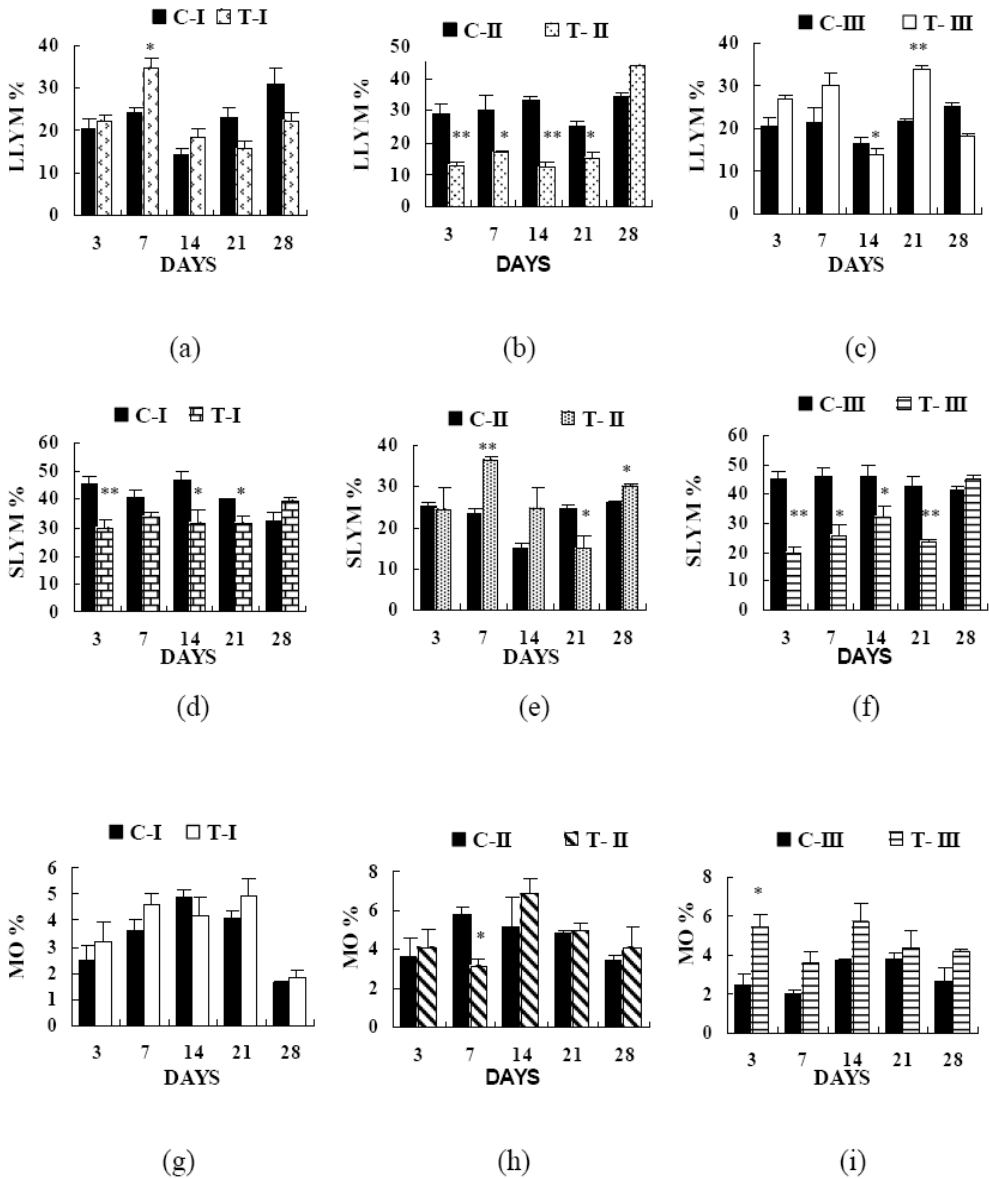


Figure 3. Changes in relative population of agranulocytes (DWBC %) viz. Large Lymphocyte (a, b, c), Small Lymphocyte (d, e, f) and Monocyte (g, h, i) respectively over time of six fish groups which were divided into 3 pairs (I-III); CI: control, TI: pesticide exposed only, CII: sham injected control, TII: Pesticide + *A. salmonicida* (MTCC 1522) exposed fish, CIII: sham injected control and TIII: Pesticide + *A. hydrophila* (MTCC 646) exposed fish; Means $\pm$ S.E.; 8 fish for each group; LLYM: Large Lymphocyte, SLYM: Small Lymphocyte and MO: Monocyte. * Significant ($P<0.05$), ** Highly significant ($P<0.01$).

B. Changes in Erythrocytic Parameters

Highly significant decrease ($P < 0.01$) of Total Erythrocyte Count (TEC) was observed initially and at the end (28 day exposure) in T-I treated group (Figure 4a) whereas significant overall increases were noticed in both T-II and T-III experimental groups (Figure 4b and Figure 4c).

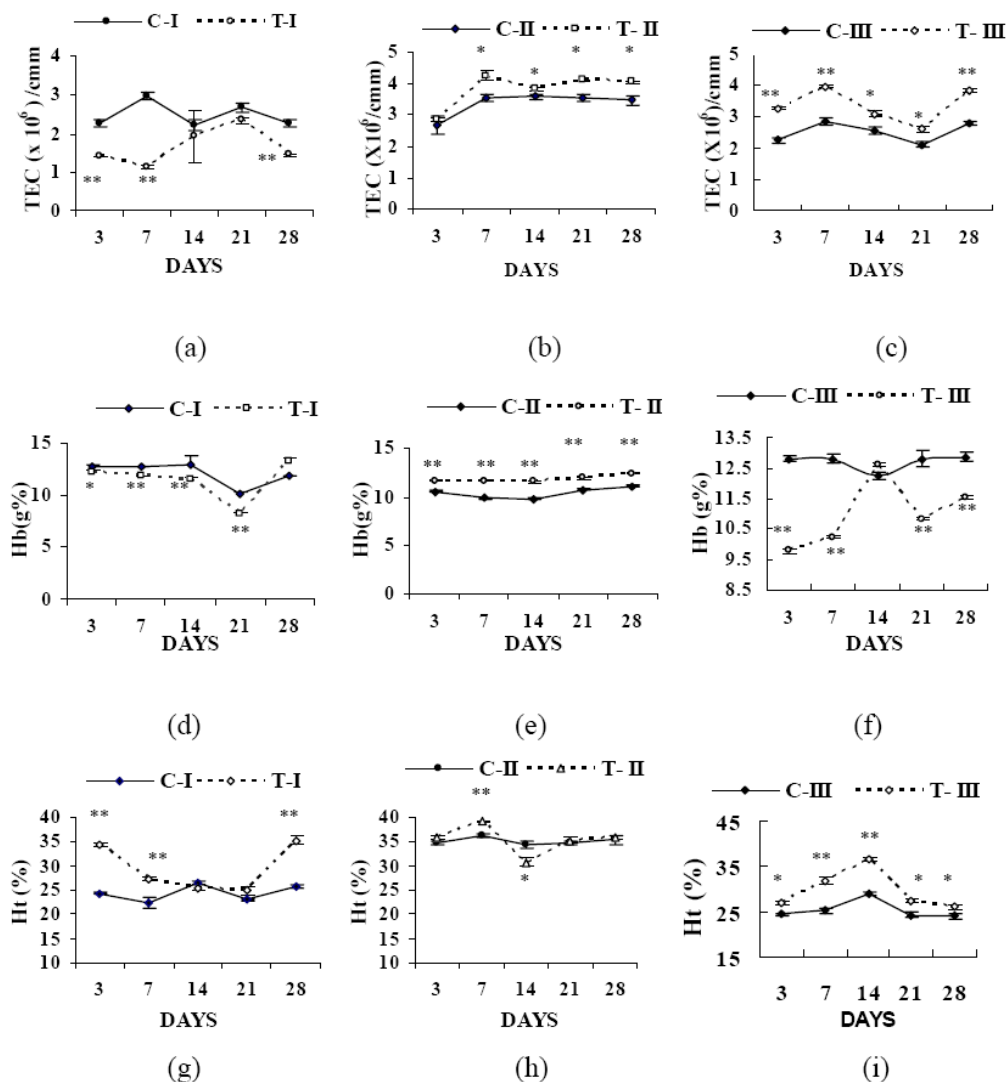


Figure 4. Changes in erythrocytic blood parameters like TEC (a, b, c), Haemoglobin (d, e, f) and Haematocrit (g, h, i) respectively over time of six fish groups which were divided into 3 pairs (I-III); CI: control, TI: pesticide exposed only, CII: sham injected control, TII: Pesticide + *A. salmonicida* (MTCC 1522) exposed fish, CIII: sham injected control and TIII: Pesticide + *A. hydrophila* (MTCC 646) exposed fish; Means $\pm$ S.E.; 8 fishes for each group; TEC: Total Erythrocyte Count, Hb: Haemoglobin and Ht: Haematocrit. * Significant ($P < 0.05$), ** Highly significant ($P < 0.01$).

Haemoglobin (Hb) values were significantly lower in T-I group up to 21 days of interval but a profound recovery was noticed after that (Figure 4d). All the Hb values were significantly high ($P<0.01$) in T-II group of fish relative to corresponding sham-injected control group over the study period (Figure 4e). In T-III group, haemoglobin values were significantly reduced except at the middle of the exposure period (Figure 4f).

In the case of Haematocrit (Ht) values, increasing differences between pesticide- exposed (T-I) and control fish population (C-I) occurred at 3, 7 and 28 days of intervals (Figure 4g).

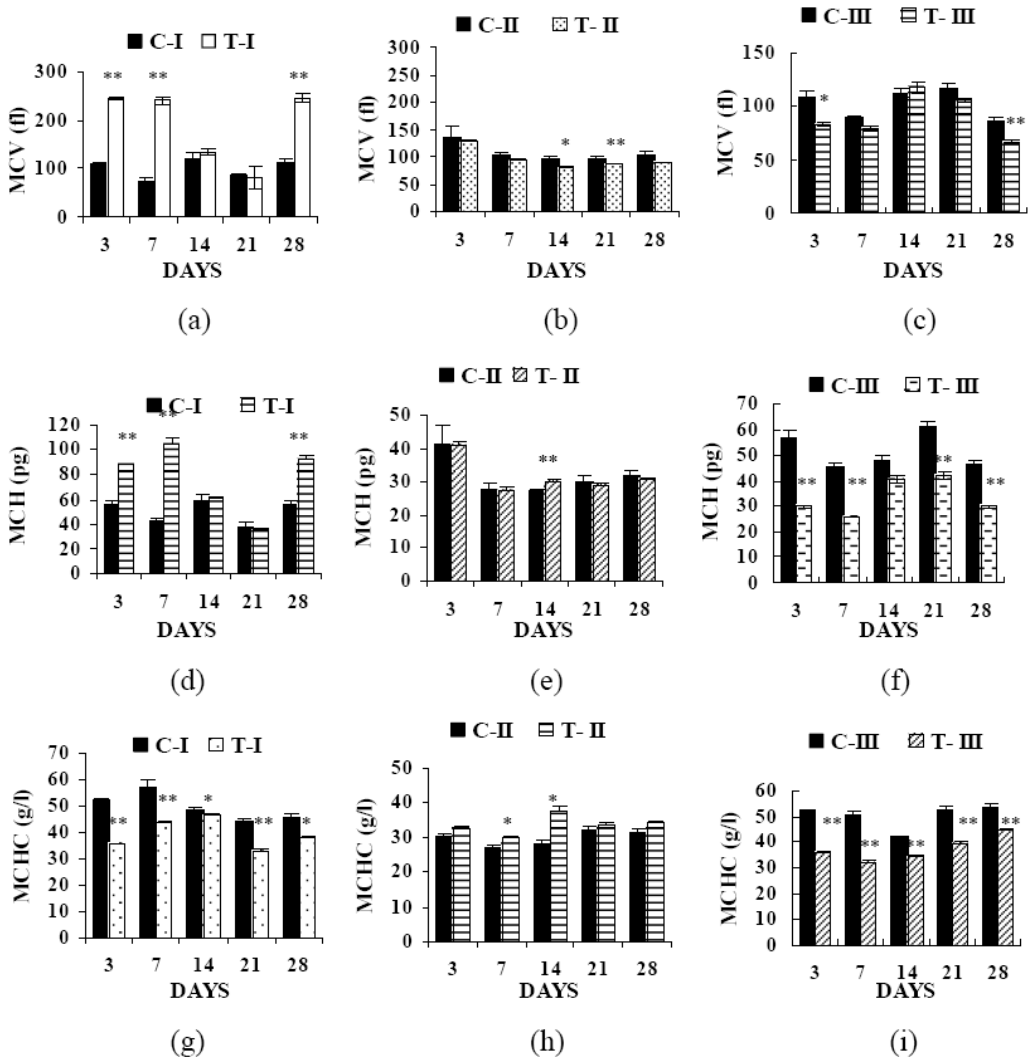


Figure 5. Changes in erythrocytic blood parameters like MCV (a, b, c), MCH (d, e, f) and MCHC (g, h, i) respectively over time of six fish groups which were divided into 3 pairs (I-III); CI: control, TI: pesticide exposed only, CII: sham injected control, TII: Pesticide + *A. salmonicida* (MTCC 1522) exposed fish, CIII: sham injected control and TIII: Pesticide + *A. hydrophila* (MTCC 646) exposed fish. Means $\pm$ S.E.; 8 fish for each group; MCV: Mean Cell Volume, MCH: Mean Cell Haemoglobin and MCHC: Mean Cell Haemoglobin Concentration. * Significant ($P<0.05$), ** Highly significant ($P<0.01$).

In T-II group, a steep increase ($P<0.01$) after 7 days of combined exposure was followed by a significant fall ($P<0.05$) in Ht value after 14 days (Figure 4h). An overall significantly increased value of haematocrit was observed over the entire study period in T-III group of fish (Figure 4i).

Highly significant increases in MCV and MCH values were observed in T-I group after 3, 7 and 28 days of exposure respectively (Figure 5a and Figure 5d). MCV was significantly reduced after 14 and 21 days (Figure 5b) but MCH was high after 21 days in T-II group (Figure 5e). A significant decrease of MCH was observed throughout the entire period in T-III group (Figure 5f) whereas low values of MCV were noticed at 3 and 28 days of intervals in this group (Figure 5c). Regarding MCHC, both T-I and T-III groups showed overall decrease at each interval from their respective controls (Figure 5g and Figure 5i) whereas T-II group of fish showed increase of MCHC value after 7 and 14 days of exposures respectively (Figure 5h).

DISCUSSION

Specific diseases of cultured fishes have been associated with the potentially stressful conditions of fish culture facilities such as handling, crowding, fluctuating water conditions (Wedemeyer, 1970; Wedemeyer and Wood, 1974). Changes in the haematology of fishes in response to stressing agents are indicators of the stressful state in fish (Iwama *et al.*, 1986) and thus are useful in deciding when to initiate prophylactic measures in fish culture. They may also provide a method of assessing the physiological status of fish stocks.

In our experiment, the leucocytic population (TLC) was reduced initially in pesticide-exposed fish than control but showed an increasing trend with time. On the other hand, leucocytic parameters (TLC and Lct) were increased initially due to neutrophilia and to some extent, eosinophilia subsequent to invasion of foreign pathogen in combined treatments. However, both type of treatments (single or combined exposure) evoked a drop in lymphocyte population (lymphocytopenia). The variable response seen in the leucocytic parameters to treatments over the sampling period can be interpreted as an initial stress-mediated reduction in leucocytes (especially lymphocytes) coupled with possible haemodilution followed by an increase in white cells, perhaps due to increase in circulating neutrophils in response to the increasing tissue damage by the pathogens. Depressed WBC counts, probably involving decreases in lymphocytes in response to stressful conditions, have been recorded in various fishes (McLeay, 1973; McLeay, 1975; Angelidis *et al.*, 1987; Reitan and Thuvander, 1994).

The depression in most of the erythrocytic parameters was also observed in fish groups treated only with pesticide except Ht, MCV and MCH values at certain exposures. The increased erythrocytic fragility as a result of pesticidal exposure or destruction of haemopoietic tissues of kidney might be responsible for this depression. On the other hand, somewhat elevated responses were noticed due to combined treatments. For instance, haemoglobin (Hb) values did not decrease in proportion to the measured falls in TEC and haematocrit, but appeared higher than expected as in Figure 4e. This apparent anomaly may be explained by the fact that haemoglobin crystals that might be formed during the progression of the disease remained in the whole blood in addition to intra-erythrocyte

haemoglobin. Such haemoglobin crystals have been early reported by Clifton-Hadley *et al.* (1987) in bacterial diseases. An analytical view of the resultant erythrocytic changes increases the possibility of employing such indices as aids in the diagnosis and assessment of fish diseases (McCarthy *et al.*, 1973; Saha *et al.*, 1997). In this study, most of the haematological changes pointed to a general haemodilution probably arising from progressive damage to the haemopoietic tissues by the pesticidal exposure alone even at a very low dose. However, the erythrocytic depression was not so much pronounced by the combined exposures probably due to elevated levels of leucocytic cell population in response to initial exposure of pesticide.

In conclusion, the work embodied in the present experiment provides thrust upon the usefulness of haemogram as pathological indications of bacterial diseases and environmental stressors in fish. This study further opens up research avenues to understand the complex nature of bacterial infections and effectual other physiological correlates.

ACKNOWLEDGMENT

The authors acknowledge University Grant Commission (UGC), New Delhi, India for financial assistance. Authors thank Dr. T. Chakrabarti of Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, IMTECH in Chandigarh, India for providing two lyophilized microbial strains of *Aeromonas* for the purpose of experimental inoculation in fish. Thanks also go to the Head, Department of Zoology, University of Calcutta for providing infrastructural facilities and Dr. P. Bag, Department of Biochemistry, University of Calcutta for technical help.

REFERENCES

- Angelidis, P., Baudin-Laurencin, F. and Youinou, P. (1987). Stress in rainbow trout, *Salmo gairdneri*: effects upon phagocyte chemiluminescence, circulating leucocytes and susceptibility to *Aeromonas salmonicida*. *J. Fish Biol.*, 31A, 113-122.
- APHA (2005). Standard Methods for the Examination of Water and Waste water (21st edition). Washington DC: American Public Health Association.
- Blaxhall, P. C. and Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *J. Fish Biol.*, 5, 771-781.
- Bols, N. C., Brubacher, J. L., Ganassin, R. C. and Lee, L. E. (2001). Ecotoxicology and innate immunity in fish. *Dev. Comp. Immunol.*, 25, 853-873.
- Bucher, F. and Hofer, R. (1993). The effects of treated domestic sewage on three organs (gills, kidney, liver) of brown trout (*Salmo trutta*). *Water Res.*, 27, 255-261.
- Burkhardt-Holm, P., Escher, M. and Meier, W. (1997). Waste-water management plant effluents cause cellular alterations in the skin of brown trout. *J. Fish Biol.*, 50, 744-758.
- Cardwell, R. D. and Smith, L. S. (1971). Haematological manifestations of vibriosis upon juvenile chinook salmon. *Prog. Fish Cult.*, 33, 232-235.
- Clifton-Hadley, R. S., Richards, R. H. and Bucke, D. (1987). Further consideration of the haematology of proliferative kidney disease (PKD) in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis.*, 10, 435-444.

- Coles, E. H. (1986). Veterinary Clinical Pathology. Philadelphia, PA: W. B. Saunders.
- Dacie, J. V. and Lewis, S. N. (1984). Practical Haematology (6th edition). Edinburg: Churchill Livingstone.
- Dunier, M. and Siwicki, A. K. (1993). Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. *Fish and Shellfish Immunol.*, 3, 423-438.
- Evenberg, D., De Graaff, P., Lugtenberg, B. and van Muiswinkel, W. B. (1988). Vaccine-induced protective immunity against *Aeromonas salmonicida* tested in experimental carp erythrodermatitis. *J. Fish Dis.*, 11, 337-350.
- Foda, A. (1973). Changes in haematocrit and haemoglobin in Atlantic salmon (*Salmo salar*) as a result of furunculosis disease. *J. Fish Res. Bd Can.*, 30, 467-468.
- Handy, R. D. and Depledge, M. H. (1999). Physiological responses: their measurement and use as environmental biomarkers in ecotoxicology. *Ecotoxicology*, 8, 329-349.
- Harbell, S. C., Hodgins, H. O. and Schiewe, M. H. (1979). Studies on the pathogenesis of vibriosis in coho salmon *Oncorhynchus kisutch* (Walbaum). *J. Fish Dis.*, 2, 391-404.
- Hesser, E. F. (1960). Methods for routine fish haematology. *Prog. Fish Cult.*, 22, 164-171.
- Iwama, G. K., Greer, G. L. and Randall, D. J. (1986). Changes in selected haematological parameters in juvenile chinook salmon subjected to a bacterial challenge and a toxicant. *J. Fish Biol.*, 28, 563-572.
- McCarthy, D. H., Stevenson, J. P. and Roberts, M. S. (1973). Some blood parameters of rainbow trout (*Salmo gairdneri* Richardson). I. The Kamloops variety. *J. Fish Biol.*, 5, 1-8.
- McLeay, D. J. (1973). Effects of ACTH on the pituitary-interrenal axis and abundance of white blood cell types in juvenile coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.*, 21, 431-440.
- McLeay, D. J. (1975). Sensitivity of blood cell counts in juvenile coho salmon (*Oncorhynchus kisutch*) to stressors including sublethal concentrations of pulp mill effluent and zinc. *J. Fish. Res. Bd Can.*, 32, 2257-2364.
- Møyner, K. (1993). Changes in serum protein composition occur in Atlantic salmon, *Salmo salar* L., during *Aeromonas salmonicida* infection. *J. Fish Dis.*, 16, 601-604.
- Mulcahy, M. F. (1969). Serum protein changes in UDN-infected Atlantic salmon: a possible method of diagnosis. *J. Fish Biol.*, 1, 333-338.
- Post, G. (1966). Serum proteins and antibody production in rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Bd Can.*, 23, 1957-1963.
- Racicot, J. G., Gaudet, M. and Leray, C. (1975). Blood and liver enzymes in rainbow trout (*Salmo gairdneri*) with emphasis on their diagnostic use: study of CCl₄ toxicity and case of *Aeromonas* infection. *J. Fish Biol.*, 7, 825-835.
- Reed, L. L. and Muench, H. (1938). A simple method of estimating fifty percent end points. *Am. J. Hyg.*, 27, 493-497.
- Řehulka, J. (2003). Haematological and biochemical analyses in rainbow trout *Oncorhynchus mykiss* affected by viral haemorrhagic septicaemia (VHS). *Dis. Aquat. Org.*, 56, 185-193.
- Řehulka, J. and Minařík, B. (2007). Blood parameters in brook trout *Salvelinus fontinalis* (Mitchill, 1815), affected by columnaris disease. *Aquaculture Res.*, 38, 1182-1197.
- Reitan, L. J. and Thuvander, A. (1994). *In vitro* responsiveness of subpopulations of leucocytes from Atlantic salmon, *Salmo salar* L., to *Aeromonas salmonicida*. *J. Fish Dis.*, 17, 549-553.

- Saha, S. K., Homechaudhuri, S. and Banerjee, S. (1997). Natural prevalence of *Trypanosoma mukundi* in *Heteropneustes fossilis* (Bloch) and related haematological analysis. *J. Aqua. Trop.*, 12(1), 87-95.
- Shieh, H. S. (1978). Changes of blood enzymes in brook trout induced by infection with *Aeromonas salmonicida*. *J. Fish Biol.*, 12, 13-18.
- Tatner, M. F. (1990). Antigen-induced blastogenesis of salmon, *Salmo salar* L., head kidney leucocytes to modified *Aeromonas salmonicida* antigens: a preliminary evaluation method for potential vaccine antigens. *J. Fish Biol.*, 36, 731-739.
- Wedemeyer, G. A. (1970). The role of stress in disease resistance of fishes. In S. F. Snieszko (Ed.), *A Symposium on Diseases of Fishes and Shellfishes* (pp. 30-35). *Am. Fish. Soc. Spec. Publ. No.5*.
- Wedemeyer, G. A. and Wood, J. W. (1974). Stress as a predisposing factor in fish diseases. *U.S. Dept. Int., Fish. Dept. Leaflet*, 38, 8.
- Wintrobe, M. M. (1967). *Clinical haematology* (6th edition). Philadelphia: Lea and Febiger.

DESCRIPTION OF TWO NEW SPECIES OF MITES INFESTING EDIBLE MUSHROOM (*PLEUROTUS OSTREATUS*)

Amrita Mukherjee¹, S. K. Gupta² and G. K. Saha^{1*}

¹Department of Zoology, University of Calcutta
35, Ballygunge Circular Road, Kolkata 7000019, India

²Formerly of Zoological Survey of India, Kolkata

ABSTRACT

Two new species each under the genus *Scutascirus* Shiba (Family: Cunaxidae) and *Gamasellodes* Atheis-Henriot (Family: Ascidae) collected from dried mushroom *Pleurotus ostreatus* in West Bengal, India are described and illustrated.

Keywords: Mushroom mite, West Bengal, New species, *Scutascirus*, *Gamasellodes*, Cunaxidae, Ascidae.

INTRODUCTION

Mites present in the mushroom growing on wheat straw causes great economic loss both in terms of quality as well as quantity. However, information available from India regarding mites infesting mushroom is very scanty. Some of the notable contributions in the relevant field include Mukherjee and Somchoudhury (1974), Das (1986), Das *et al.* (1987a, 1987b), Das and Mishra (1995), Jhansi Rani (2001) and Bhattacharyya (2003) etc. while those from abroad are Clift and Terras (1993), Wu and Zhang (1993), Ferragut *et al* (1997) and Okabe-kimiko (1999) etc. As far as information available, about 50 species of mites are known to infest mushroom throughout the world. During the course of study on stored product mites of West Bengal two species one each under genus *Scutascirus* and *Gamasellodes* have been recorded from the edible mushroom *Pleurotus ostreatus* as new species. These two species are described and illustrated in the present communication.

* Corresponding Author: Dr. G. K. Saha, *Email: gkszoo@rediffmail.com /gkszoo@gmail.com

MATERIALS AND METHODS

The mites were collected with the help of a Berlese-Tullgren funnel extraction apparatus. Specimens were cleared in lactic acid. Mites were then directly mounted in Heinz’s medium. All the measurements are in micron. The types of two new species are presently deposited in the collection of the Entomology Laboratory, University of Calcutta, to be deposited in due course in the National Collection of the Zoological Survey of India, Kolkata.

RESULT

FAMILY 1: CUNAXIDAE Thor

Genus : *Scutascirus* Shiba
Scutascirus ramakrishnai sp. nov.(Figures 1-7)

Description of Female

Hypostome almost rectangular. Palp five segmented; trochanter without any seta; femur and genu bear one dorsal seta each, tibia has two lateral setae and one ventral seta. Tarsus bears five setae including one long seta at the tip. Palp tibiotarsal claw present apically. Propodosomal shield not reticulate but slightly rugose and extends upto posterior tip of the body. Body 330 long, 215 wide (maximum width). Propodosomal shield with two pairs of setae in addition with two pairs of sensory setae measuring, anterior sensillum –110, posterior sensillum –96,P₁-17, P₂-20.Measurement of hysterosomal setae; D₁-20,D₂-13, D₃-17,D₅-20,L₁-14,LS-17.Setae D₁-D₄ and L₁ and L₂ situated on shield. Hysterosomal shield tranversely reticulated posteriorly, anterior reticulation pattern not discernible. Ventral shield weakly developed. Striation pattern longitudinal medially, irregular laterally and circular posteriorly (as figured). Ventral setae minute. The number of the setae on different leg segments are as given in the table-1.

Table 1. The number of the setae on different leg segments

Leg Parts	Leg I	Leg II	Leg III	Leg IV
Trochanter	2 dorsal	2dorsal	4dorsal	1dorsal
	2ventral	2ventral	2ventral	
Femur	2Dorsal	2dorsal	2dorsal	1dorsal
	2ventral	1ventral	1ventral	1ventral
Genu	2dorsal	2dorsal	2dorsal	4dorsal
	2ventral	1ventral	2ventral	3ventral
Tibia	3dorsal	4dorsal	3dorsal	3dorsal
	1ventral	1ventral	2ventral	1ventral
Tarsus	10dorsal	10dorsal	7dorsal	10dorsal
	2ventral	2ventral	4ventral	4ventral

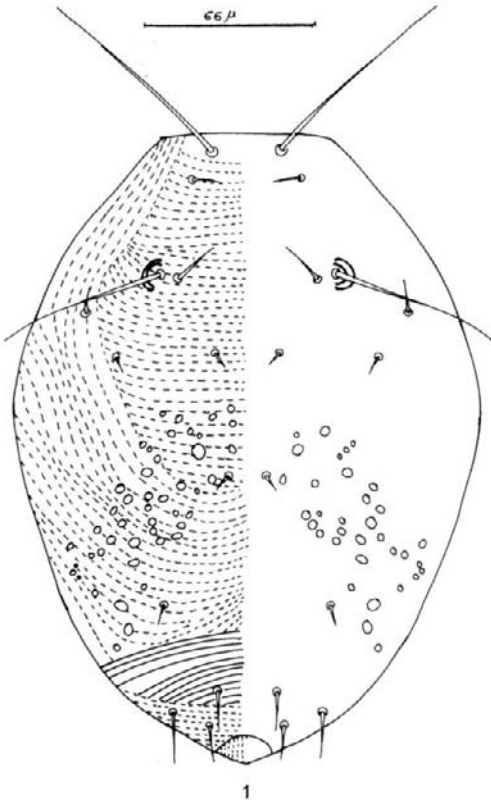


Figure 1. Dorsal view.

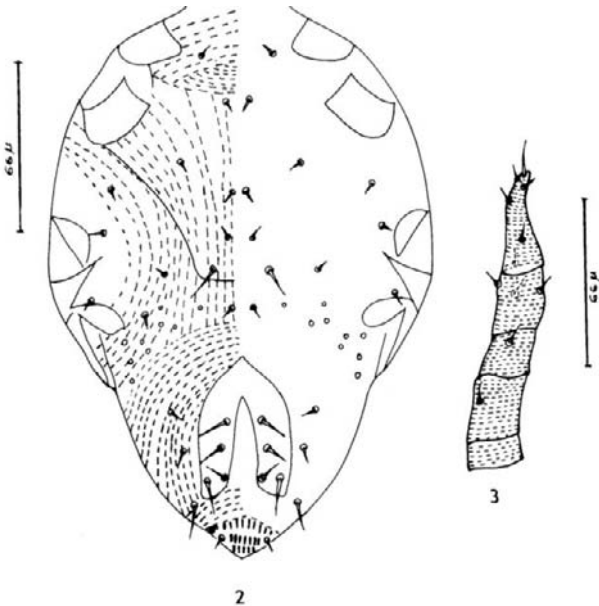


Figure 2. Ventral view.

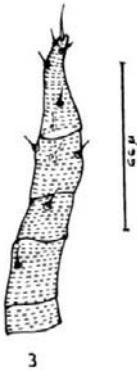


Figure 3. Palp.

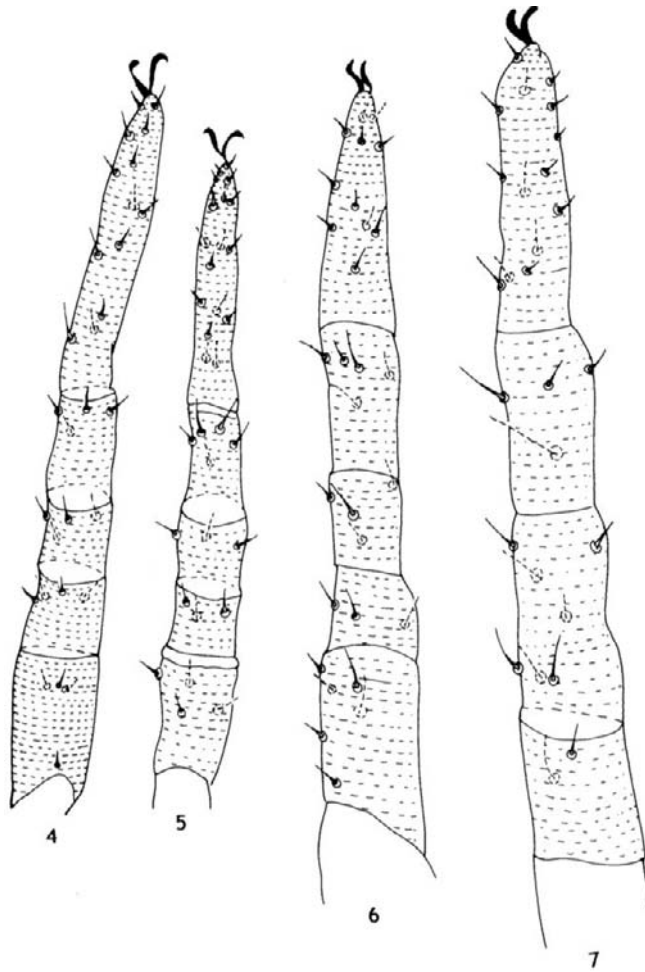


Figure 4. Leg I. Figure 5. Leg II. Figure 6. Leg III. Figure 7. Leg IV.

All tarsi end in a claw. Trichobothria on leg IV measures-43. Measurement of leg; LegI-244, LegII-238, LegIII-271, Leg-IV-287. Genital plate with four pairs of genital setae, paragenital setae two pairs.

Male unknown.

Material Examined

Holotype female, India, West Bengal, Narendrapur, South24 Paraganas district. ex. dried mushroom; 5.12.2007; Coll. Amrita Mukherjee.

Paratype-1 female, date same as per holotype.

Etymology: The naming has been made after Lord Ramakrishna whose temple is located at the collection area.

Discussion: - This new species resembles *Scutascirus exasperatus* (Shiba, 1978) but the species differs from it, in chaetotaxy of palp, in leg chaetotaxy having more number of setae in all leg segments as compared to *Scutascirus exasperatus* (Shiba, 1978). Ventral hysterosomal setae also relatively shorter in this new species.

FAMILY 2: ASCIDAE Voigts and Oudemans

Genus *Gamasellodes* Athies-Henriot

Gamasellodes ostreatus sp. nov. (Figures 8-11)

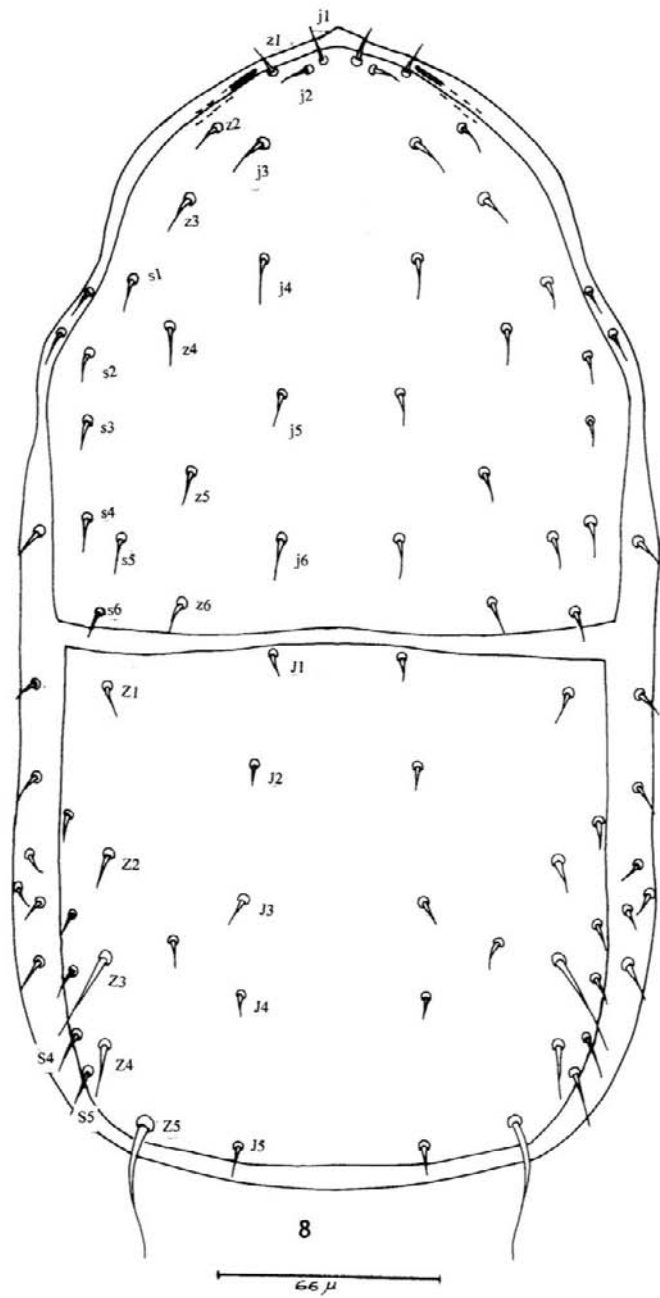


Figure 8. Dorsal view.

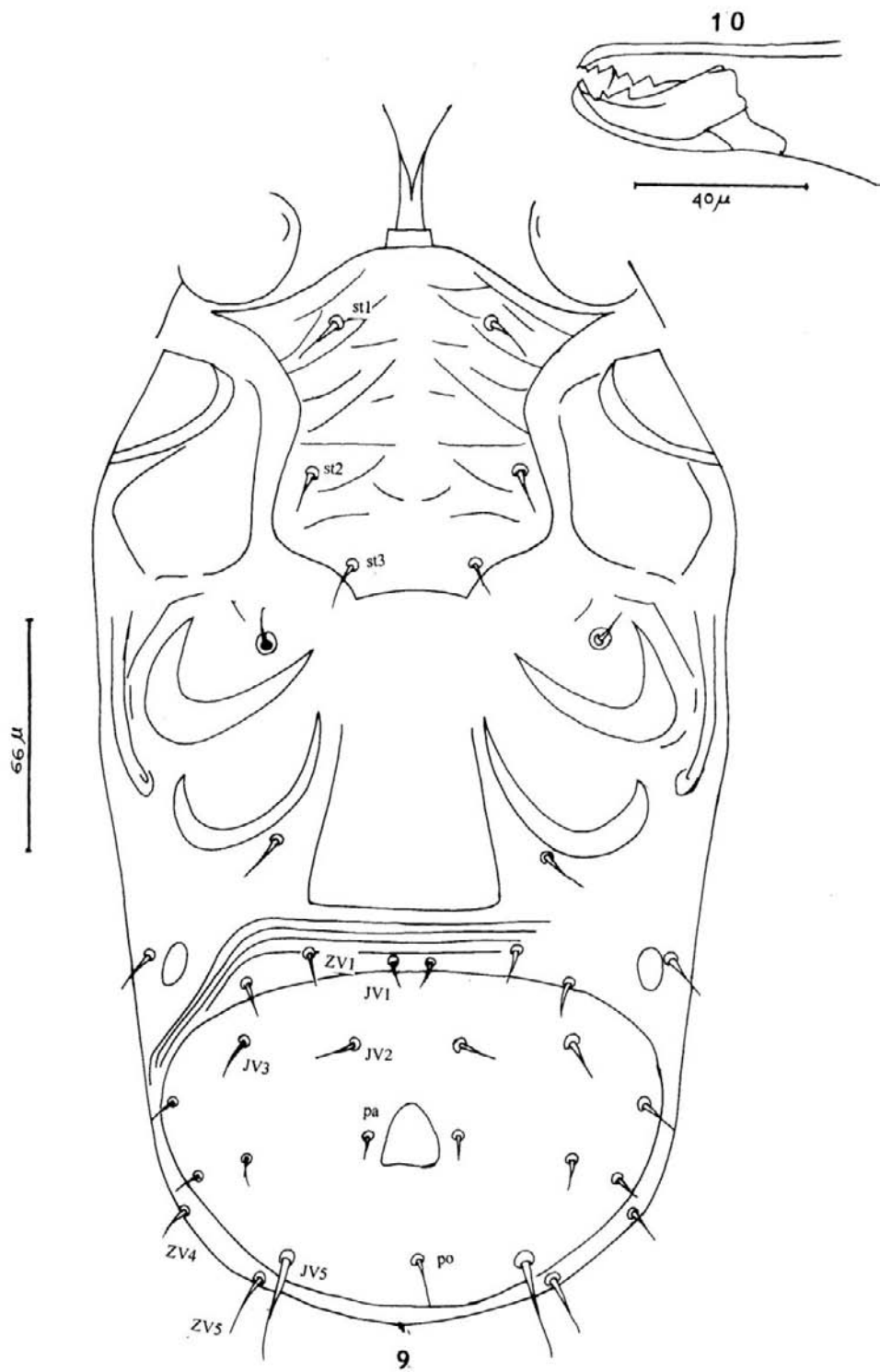


Figure 9. Ventral view. Figure 10. Chelicera.

Description of Female

Dorsum 297 long and maximum width 149 with two dorsal shields, anterodorsal shield 149 long, 142 wide, the shield smooth having eighteen pairs of setae. The setae measure; j1-13, z1-17, s5-14. Posterodorsal shield 122 long, 132 wide with fifteen pairs of setae measuring; Z4-30, Z5-40, S4-26, S5-33. Interscutal membrane with three pairs of setae at the anterodorsal region whereas R1, R2, R3, R4 and R5 at the posterodorsal region. Tritosternum 33 long with a pair of lacini. Sternal shield 99 long, 66 wide, shaped as figured, posterior margin of sternal shield slightly concave with three pairs of sternal setae st1, st2, st3 measuring 13 long. Metasternal shield oval shaped with a seta st4 measuring 10 long. Genital shield 50 wide with a seta on either side measuring 13 long. Ventrianal shield 89 long, 116 wide with seven pairs of setae measuring; JV5-33, JV4-27 with pair of pre-anal setae pa measuring, 20 long and a post anal seta po measuring, 23 long. Five pairs of setae present around ventrianal shield Metapodal plate elongated oval and 10 long. Ventrianal shield weakly developed transversely striated on the anterior portion. The area between genital and ventrianal shield is transversely striated medially and longitudinally striated laterally. Corniculi parallel. Fixed digit on chelicera with four sharp teeth, with a fine *pilus dentilis*. Movable digit on chelicera 26 long with two teeth. Leg I –IV with ambulacra and a pair of claws. Legs measured-Leg I –224, Leg II – 168, Leg III – 132, Leg IV – 224. Leg chaetotactic formula-

2 2

Genu II- 1 — —1

0 1

2 2

Tibia II - 3 — — 1

2 2

2 1

Genu III - 2 — —1

1 0

2 1

Tibia III- 2 — — 1

1 1

Leg IV chaetotaxy as illustrated (figure no.11).

Male unknown

Material Examined

Holotype female, India, West Bengal, Narendrapur, South24 Paraganas district, ex. dried mushroom. 5.12. 2007. Coll. Amrita Mukherjee.

Paratype-1 female, date same as per holotype.

Etymology: This new species has been named after the species of mushroom *Pleurotus ostreatus* wherefrom it was collected.

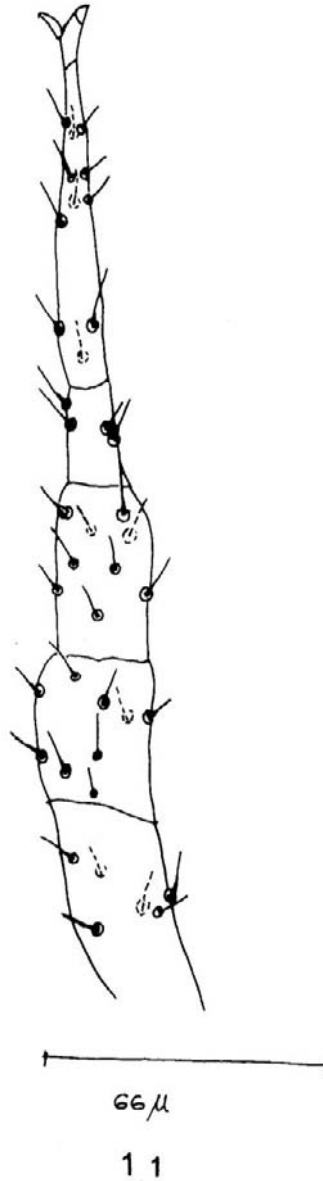


Figure 11. Leg IV.

DISCUSSION

This new species differs from related species *G. andhraensis* (Bhattacharyya, 2003) on the following points:

1. In this new species anterodorsal shield with eighteen pairs of setae whereas fifteen pairs present in *G. andhraensis*.
2. The setal length with respect to Z4 and Z5 in both the specimen differs as Z4 and Z5 measure 30 and 40 long respectively in new species while in *G. andhraensis* Z4-23, Z5-37
3. The length of preanal setae on ventrianal shield vary in both the species with regard to their lengths and number.
4. The lateral interscutal setae also differ in both the species in their numbers.

This new species differs from related species *G. spinosus* (Bhattacharyya and Sanyal, 2002) on the following points:

1. It differs from *G. spinosus* in having eighteen pairs of anterodorsal setae
2. Difference in the number and position of setae in the interscutal membrane around ventrianal shield.
3. Posterior margin of sternal shield highly concave in *G. spinosus* whereas slightly concave in this new species.

This new species differs from related species *G. islandicus* (Bhattacharyya and Sanyal, 2002) on the following points:

1. In the number of setae on the anterior dorsal shield, seventeen pairs in *G. islandicus* and eighteen pairs in new species.
2. In the number of setae on lateral interscutal membrane.
3. In having six teeth on fixed digit of chelicera against four in case of new species.
4. Sternal shield one and half time as long as wide in new species and as long as wide in *G. islandicus*.
5. Metasternal shield absent in *G. islandicus* and distinctly present in the new species.

This new species differs from another related species *G. sternalis* (Bhattacharyya and Sanyal, 2002) on the following points:

1. Lacking small platelates around ventrianal shield.
2. In measurement of dorsal idiosomal setae
3. Metapodal plates two pairs in *G. sternalis* and one pair in new species.

This new species differs from another related species *G. adrianae* (David Evans Walter, 2003) on the following points:

1. In number and position of dorsal idiosomal setae.
2. In dentition on chelicera.
3. In setal number on Ventrianal shield.

Considering distinct differences in number, size and disposition of setae in different body parts and in other features the species could be considered as a distinct undescribed species under the genus *Gamasellodes*.

ACKNOWLEDGMENT

Authors are grateful to the Head, Dept. of Zoology, University of Calcutta for providing laboratory facilities.

REFERENCES

- [1] Bhattacharyya A. K. (2003). Two new species of Ascidae (Acarina: Mesostigmata) from India. *Zootaxa*. 189 : 1-10.
- [2] Bhattacharyya A. K. and Sanyal A. K. (2002). Three new species and some new records of the genus *Gamasellodes* Athias-Henriot (Acarina:Ascidae) from India. *Acarologia*.3:229-238.
- [3] Das P. (1986). *Bionomics and control of mushroom mites*. Ph. D. Thesis. BCKV.
- [4] Das P. Somchoudhury A.K. and Mukherjee A.B.(1987a) Rearing of mushroom mites on artificial diets. *Environment and Ecology*.5(4): 685-687.
- [5] Das P. Somchoudhury A.K.and Mukherjee A. B. (1987b). Nature and habitat of mushroom mites. *Environment and Ecology*. 5(4): 677-680.
- [6] Clift A. D. and Terras M.A.(1993).The relative efficacy of insecticides and disinfectants against mushroom mites. *Gen. App. Ento*.24: 64pp.
- [7] Das T. K. and Mishra D. N. (1995). Toxicity of some synthetic pyrethroids of *Rhizoglyphus echniopus* (Acarina:Acaridae) a pest of mushroom. *Environment and Ecology*. 13(2):366-368.
- [8] David Evans Walter. (2003). The genus *Gamasellodes* (Acari:Mesostigmata:Ascidae) : New Australian and North American species. *Systematic and Applied Acarology special publication*. 15 :1-10.
- [9] Ferragut F. Gea F. J. and Garcia Morras J.A. (1997). The mushroom mite *Brennandania lambi* (Krczai) Bolotin-de-sanidadvegetal, *PlagAs*.23(2):301-311.
- [10] Jhansi Rani, B. (2001). Insect pest management in mushrooms. National Seminar on Tropical mushroom production and export. Bangalore. p. 96-99.
- [11] Mukherjee A. B. and Somchoudhury A.K. (1974). Mite pest on mushroom. *FAO Plant Protection Bulletin*. 22: 51pp.
- [12] Okabe-Kimiko.(1999).Vectoring of *Hypocrea nigricans* (Hypocreales:Hypocreacea). *Experimental and Applied Acarology*. 23(8): 653-658.
- [13] Shiba, M. (1978) Taxonomic investigation on free living prostigmata from Malay peninsula. *Nat and Life in S.E. Asia*.7: 83-229.
- [14] Wu J. and Zhang Z. Q. (1993) Host feeding damage and control of the mushroom pest, *Brennandania lambi* (Acari: Pygmiphoridae) in China. *Experimental and Applied Acarology*. 17 (3): 233-240.

USEFULNESS OF NONINVASIVE METHODS OF DNA SAMPLING BUT WITH A CAUTION

***Ibrahim A. Arif, Haseeb A. Khan*, Ali A. Al Homaidan,
Ahmad H. Al Farhan, Ali H. Bahkali, Mohammad Shobrak and
Mohammad Al Sadoon***

Molecular Fingerprinting and Biodiversity Unit,
Prince Sultan Research Chair for Environment and Wildlife,
College of Sciences, King Saud University, Riyadh, Saudi Arabia

ABSTRACT

The dependency on noninvasive sources of DNA such as feces, hair and feathers for genetic analyses is particularly important in studies where conventional sampling is hard to practice, as in the case of wildlife animals. In view of earlier reports, we hereby address some important issues on merits, demerits and precautions related to the use of noninvasive methods of DNA sampling for molecular markers analysis.

Keywords: DNA, Noninvasive methods, Molecular markers, Fingerprinting, Conservation, Wildlife, Endangered animals.

Use of noninvasive sources of DNA such as feces, hair and feathers for genetic analyses is particularly important in studies where conventional sampling is difficult due to animal size, behavior, conservation status and ethical considerations. Capturing of wild animals for sampling often causes a considerable amount of stress. Nocturnal animals, marine mammals or animals living in dense forest are particularly difficult to sample because they are hard to trace. Recently, noninvasive methods of collection of biological samples have been successfully employed for population estimation of wildlife species (Piggott et al 2006, Bhagavatula and Singh 2006, Prugh et al 2005, Kurose et al 2005). Although the technique can be potentially useful for wildlife animals, there are some disadvantages such as small amount of DNA present in the feces or the DNA can be highly degraded due to passing

* Correspondence to: Haseeb Ahmad Khan PhD, MRACI (Aus), FRSC (UK) Chair Professor, College of Science, Bld 5, Room 2B42, King Saud University. P.O. Box 2455, Riyadh 11451. Saudi Arabia. E-mail: khan_haseeb@yahoo.com

through the intestinal track. Some of the important issues related to noninvasive methods of DNA sampling for molecular markers analysis are summarized in Table 1.

Table 1. Important noninvasive sources of DNA and their merits/demerits

Source	Taxa	Marker	Remark	Reference
Feces	Indian leopard	12S rRNA	For field analysis, authentication of collected scat is necessary before analysis.	Pandey et al 2007
Feces	Oryx	CR	No difference in the quality of DNA between droppings and tissue samples.	Masembe et al 2006
Feces	Oryx	CR, SSR	For fecal samples, heterozygous genotypes were repeated twice and homozygous were repeated 4-6 times. Failure to amplify large product with fecal DNA.	Iyengar et al 2007
Feces	Andean cat	16S rRNA	Fecal DNA is often of low quality. Short segments of genes should be selected. Hairs and other undigested rests from preys present on fecal samples may interfere and produce hybrid patterns.	Cossios and Angers 2006
Feces	Giant otter	CR, Cyt b	Fecal DNA is not suitable for microsatellites and intron sequencing.	Garcia et al 2007
Feces	Bighorn sheep	SSR	With only outer pellet material, PCR was excellent. When inner pellet material was included PCR success declined. Fecal pellets of herbivore species that live in wet environments may undergo more DNA degradation if not collected fresh.	Wehausen et al 2004
Feces	Bengal tiger	SSR	The fecal samples collected from captive tigers were amplified with 100% success versus 60% with fecal samples from field.	Bhagavatula and Singh 2006
Feces	Leopard cat	Cyt b, SRY	It is sometimes difficult to analyze long fragments using fecal samples, because fecal DNA is often fragmented due to time process and environmental conditions.	Kurose et al 2005
Feces	Brown bear	Sex determine	When female carnivores consume male mammalian prey, all are incorrectly scored as male due to amplification of X and Y chromosome fragments.	Murphy et al 2003
Feather	Kakerori (bird)	SSR	Success in developing microsatellite markers from feather samples.	Chan et al 2008
Feather	Harpy eagle	SSR, Sex determine	The sex determination markers easily and consistently differentiated males from females even with highly degraded DNA extracted from naturally shed feathers.	Banhos et al 2008
Feather	Pigeon	CR	Risk of repeated sampling from the same individuals. High rate of genotyping error due to low DNA quantity and quality.	Seki 2006
Feather	Birds	SSR	PCR success is higher from extracts of plucked feathers than moulted feathers.	Segelbacher 2002

Abbreviations are: CR, control region; Cyt b, cytochrome b; SSR, short sequence repeat; SRY, sex-determining region Y.

Garcia et al (2007) have suggested that the noninvasive samples such as feces and mucus for molecular genetic analyses are informative DNA sources to analyze giant otter (*Pteronura brasiliensis* populations (Garcia et al 2007). The mucus quality and amount obtained from feces depends on the sample age; when feces are fresh it is possible to collect only the mucus using a syringe without needle. When feces are old or dry, the surface is scraped off targeting epithelial cells sloughed from the gut lining during gut passage of the fecal bolus (Garcia et al 2007). Wehausen et al (2004) have got best results while using 60 mg of scraped outer pellet material from Bighorn sheep feces whereas the use of 30 mg caused a 54% decline in signal strength, though PCR success was little affected suggesting that the outer pellet layer contained a significant amount of high quality template DNA. The fecal samples collected from captive tigers have been amplified with 100% success as compared to 60% amplification success with fecal samples from the field; moreover the rate of errors generated from captive animal fecal samples is considerably lower than the errors generated from fecal samples collected from the jungle (Bhagavatula and Singh 2006). There is a potential concern over using fecal DNA from herbivorous animals because of inhibitory effects of plant secondary compounds on PCR reactions; consequently the amplification success depends on maximizing the concentration of DNA from target animal while minimizing secondary compounds originating from dietary plant materials (Fernando 2003). Error in sex identification using fecal DNA of carnivorous animals would result if they have consumed prey of opposite sex (Murphy et al 2003). Moreover, it is sometimes difficult to analyze long fragments of DNA with fecal samples because fecal DNA is often degraded due to the time process and environmental conditions (Kurose et al 2005).

Recently, molted feathers have been used as noninvasive samples for studies on the genetic structure of birds (Horvath et al 2005, Seki 2006). Unfortunately, molted feather samples might result a genotyping error due to low DNA quality and quantity as well as poor extraction yields (Taberlet et al 1999). Segelbacher (2002) have observed that only 50% of molted feather samples are reliably genotyped. Genomic DNA has been extracted from a 1 cm segment at the root end of feathers (Hoglund et al 2007). A feather with a rachis <0.2 mm in diameter may contain several hundred pulp cells, corresponding to roughly 10^5 - 10^6 copies of mtDNA, 10^4 - 10^5 copies of nuclear ribosomal DNA and 10^2 - 10^3 copies of single locus nuclear genes hence the amplification can be carried out on both mitochondrial and nuclear DNA (Taberlet and Bouvet 1991). Although blood and tissues are the most commonly used sources of DNA for isolating microsatellites, recently Chan et al (2008) have successfully used the feather DNA for developing microsatellite markers for the endangered forest bird, kakerori (*Pomarea dimidiata*).

In conclusion, although the DNA obtained from noninvasive sources such as feces and feathers may not be of such high quality as extracted from fresh blood or tissue samples, it offers a more convenient and practically feasible alternative for fingerprinting of wildlife animals. However, special care and caution should be undertaken at sample collection, DNA extraction and analysis levels while working with fecal or feather specimens to avoid spurious results and to achieve high level of accuracy and reproducibly.

REFERENCES

- Banhos A, Hrbek T, Gravena W, Sanaiotti T, Farias IP. Genomic resources for the conservation and management of the harpy eagle (*Harpia harpyja*, Falconiformes, Accipitridae). *Genet. Mol. Biol.* 2008;31:146-154.
- Bhagavatula J, Singh L. Genotyping faecal samples of Bengal tiger *Panthera tigris tigris* for population estimation: A pilot study. *BMC Genetics* 2006;7:48.
- Chan CH, Zhao Y, Cheung MY, Chambers GK. Isolation and characterization of microsatellites in the kakerori (*Pomarea dimidiata*) using feathers as source of DNA. *Conserv. Genet.* 2008;9:1067-1070.
- Cossios D, Angers B. Identification of Andean felid feces using PCR-RFLP. *Mastozool. Neotrop.* 2006;13:239-244.
- Fernando PT, Vidya NC, Pajapakse C, Dangolla A, Melnick DJ. Reliable noninvasive genotyping: fantasy or reality. *J. Hered.* 2003;94:115-123.
- Garcia DM, Marmontel M, Rosas FW, Santos FR. Conservation genetics of the giant otter (*Pteronura brasiliensis* (Zimmerman, 1780)) (Carnivora, Mustelidae). *Braz. J. Biol.* 2007;67:819-827.
- Hoglund J, Larsson JK, Jansman HA, Segelbacher G. Genetic variability in European black grouse (*Tetrao tetrix*). *Conserv. Genet.* 2007;8:239-243.
- Horváth MB, Martínez-Cruz B, Negro JJ, Kalmár L, Godoy JA. An overlooked DNA source for non-invasive genetic analysis in birds. *J. Avian. Biol.* 2005;36:84-88.
- Iyengar A, Gilbert T, Woodfine T, Knowles JM, et al. Remnants of ancient genetic diversity preserved within captive groups of scimitar-horned oryx (*Oryx dammah*). *Mol. Ecol.* 2007;16:2436-2449.
- Kurose N, Masuda R, Tatara M. Fecal DNA analysis for identifying species and sex of sympatric carnivores: A noninvasive method for conservation on the Tsushima Islands, *Jpn. J. Hered.* 2005;96:688-697.
- Masembe C, Muwanika VB, Nyakaana S, Arclander P, Siegmund HR. Three genetically divergent lineages of the Oryx in eastern Africa: evidence for an ancient introgressive hybridization. *Conserv. Genet.* 2006;7:551-562.
- Murphy MA, Waits LP, Kendall KC. The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*). *Mol. Ecol.* 2003;12:2261-2265.
- Pandey PK, Dhotre DP, Dharne MS, et al. Evaluation of mitochondrial 12S rRNA gene in the identification of *Panthera pardus fusca* (Meyer, 1794) from field-collected scat samples in the Western Ghats, Maharashtra, India. *Curr. Sci.* 2007; 92:1129-1133.
- Piggott MP, Banks SC, Stone N, Banffy C, Taylor AC. Estimating population size of endangered bush-tailed rock-wallaby (*Petrogale penicillata*) colonies using faecal DNA. *Mol. Ecol.* 2006;15:81-91.
- Prugh LR, Ritlands CE, Arthur SM, Krebs CJ. Monitoring coyote population dynamics by genotyping faeces. *Mol. Ecol.* 2005;14:1585-1596.
- Segelbacher G. Noninvasive genetic analysis in birds: testing reliability of feather samples. *Mol. Ecol. Notes* 2002;2:367-369.
- Seki SI. Application of molted feathers as noninvasive samples to studies on the genetic structure of pigeons (Aves: Columbidae). *J. For Res.* 2006;11:125-129.

-
- Taberlet P, Bouvet J. A Single Plucked Feather as a Source of DNA for Bird. *Genet. Stud. Auk*. 1991;108:959-960.
- Taberlet P, Waits LP, Luikart G. Noninvasive genetic sampling: look before you leap. *Trends Ecol. Evol.* 1999;14:323-327.
- Wehausen JD, Ramey RR, Epps, CW. Experiments in DNA Extraction and PCR Amplification from Bighorn Sheep Feces: the Importance of DNA Extraction Method. *J. Hered.* 2004;95:503-509.

THYROID HORMONE MEDIATED MAINTENANCE OF GLUCOSE HOMEOSTASIS AND CYTOMORPHOLOGY OF CARDIAC MUSCLE AND SKIN IN RAT

***Sudipta Chakrabarti[#], Srikanta Guria[#], Nisha Balmiki and
Madhusudan Das^{*}***

Department of Zoology, University of Calcutta
35 Ballygunge Circular Road
Kolkata-700 019, India

ABSTRACT

Thyroid hormones promote normal growth and development and regulate a number of homeostatic functions in the body (Fujita, 1988). We hypothesized thyroid hormone may have an important role in maintaining the glucose homeostasis and morphology of cardiac muscle and skin in rat. We used methimazole 20 mg/kg and thyroxine 600 µg/kg body weight/day for 14 days intraperitoneally to the experimental groups of rat to induce hypo- and hyperthyroid like conditions respectively. Serum T₃, T₄ hormones were measured by radioimmunoassay (RIA). Serum Glutamic Oxaloacetate Transaminase (SGOT) or Aspartate Amino Transferase (AST) measured by Spectrophotometric method. T₃ and T₄ levels were significantly reduced in hypothyroid rat but increased in hyperthyroid rat as expected. Thyroxine induced hyperthyroid rat exhibited hypertrophy in heart muscle and elevation of serum SGOT level.

Both hypo- and hyperthyroid induction changes the glucose homeostasis as reflected in Insulin Tolerance Test (ITT). The elevated blood glucose level returned to the basal level after 120 minutes of insulin challenge in control rat. But the same does not happened in hypo- and hyperthyroid rat. Consequently methimazole induced thyroid hormone deprivation also changes the skin architecture.

Keywords: hypothyroid, hyperthyroid, methimazole, thyroxine.

[#] Equal contributor

^{*} Corresponding author: Phone: 91-9831281756; e-mail address: madhuzoo@yahoo.com

INTRODUCTION

The thyroid gland is specialized organ for endocrine function in the body. The gland secretes thyroid hormones, primarily 3,5,3',5'-l-tetraiodothyronine (T₄), and a lesser quantity of 3,5,3'-l-triiodothyronine (T₃). Thyroid hormones promote normal growth and development and regulate a number of homeostatic functions in the body (Fujita, 1988). The recent research revealed that thyroid abnormality is the source of the many other organ disorders (Dimitriadis *et al.*, 1991; Hoppner *et al.*, 1989). But it is not very much clear how thyroid deregulation exerts its effect on the function of other organs in the body.

Earlier research revealed the possible mode of regulatory action of thyroid hormone (TH) in heart which direct delayed action at the level of gene expression (Segal, 1996). Administration of l-thyroxine (T₄) 10 µg/animal per day, lead to 30% increase in total heart weight and a 40% increase in the myosin content of the *in situ* heart when compared with control (Klein and Hong, 1986). Serum glutamic oxaloacetate transaminase (SGOT) or aspartate amino transferase (AST) is found in every tissue of the body, including red blood cells and is particularly high in the cardiac muscle. Thyroid hormone status is also important for glucose homeostasis (Chakrabarti *et al.*, 2007). Impaired glucose tolerance is a frequent complication of hyperthyroidism. Despite immense investigations, the role of thyroid gland in glucose homeostasis has remained elusive. We hypothesized thyroid hormone may have an important role in maintaining the glucose homeostasis, and morphology of cardiac muscle and skin in rat.

Therefore, we attempted to uncover this complex interaction by generating hypothyroidism (by treatment with Methimazole) and hyperthyroidism (by treatment with thyroxine) rats and observed the effects on glucose levels during Insulin Tolerance Test (ITT). We measured the serum T₃ and T₄ hormones level to confirm induction of hypo and hyperthyroid like conditions. Serum SGOT level was measured to test whether it changes with the cardiac hypertrophy. Consequently we also investigated the histological changes of cardiac muscle and skin.

MATERIALS AND METHODS

The laboratory experiments were performed using rat (*Rattus rattus*). Normal young adult rat aged 8-10 weeks and weight 100-110 g were housed in polypropylene cages and were acclimatized in laboratory condition for a week with natural light and dark schedules prior to experimentation. The animals were fed standard rodent diet and water was provided *ad libitum*. They were divided into hypothyroid, hyperthyroid and their respective control groups. Group I animals were treated with Methimazole (Sigma, USA) 20 mg/kg body weight/day for 14 days. Group II animals were treated with Thyroxine (Glaxo, India) 600 µg/kg body weight/day for 14 days. Before autopsy, Rats were anesthetized with chloroform. Blood was drawn directly from the heart for serum T₃, T₄ hormones and SGOT. Heart and dorsal skin were dissected out and fixed in Bouin's fixative (Parakkal, 1961). Tissues were embedded in paraffin, sectioned (5 µm), mounted on glass slide and stained with hematoxylin-eosin for histological analysis. T₃ or T₄ concentrations in rat serum were determined by radioimmunoassay (RIA). ¹²⁵I-labeled thyroid hormone (either T₃ or T₄)

competes with thyroid hormone in the serum sample and for antibody sites on the tube, in the presence of blocking agents for thyroid hormone binding proteins. For glucose estimation blood was collected first from the tail veins of control and treated (hypo- and hyperthyroid) rats after 18 hours of fasting. Insulin was injected intraperitoneally then blood samples were collected at different time points. 0 minute, 30 minutes, 60 minutes, 90 minutes and 120 minutes of blood glucose was measured using a blood glucose monitoring system (ACON Laboratories, Inc. San Diego, USA). Serum (SGOT) was measured following the principle of transamination. The substrates in the reaction are a ketoglutaric acid plus L-aspartate. The products formed by enzyme action are glutamate and oxaloacetate. A red colour was produced on the addition of sodium hydroxide. The intensity of colour is related to the enzyme activity (Reitman and Frankel, 1957).

RESULTS

Serum T₃ and T₄ Level

The results showed significant changes in T₃, T₄ hormones (Figure 1A and B). The serum T₃ and T₄ levels were significantly reduced in hypothyroid rat whereas in contrast the levels significantly elevated in hyperthyroid rat as expected and confirming that they were indeed in a hypothyroid and hyperthyroid state.

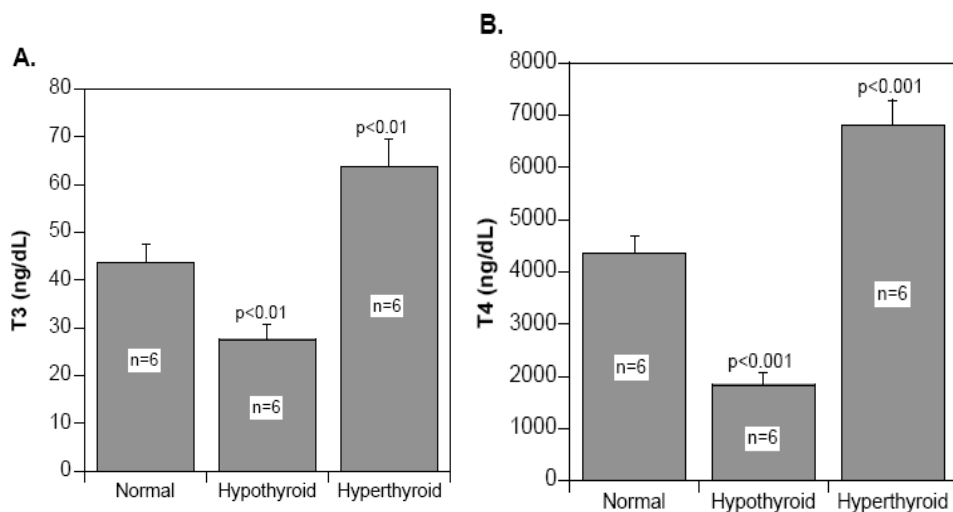


Figure 1. Plasma concentrations (ng/dL) of T₃ (A) and T₄ (B) in hypothyroid and hyperthyroid rats. Values are expressed as Mean $\pm$ SEM.

Histology of Skin

Significant changes were observed in the cytomorphology of skin in hypothyroid group when compared with the control group under light microscope (picture not shown). General morphology of skin was altered in hypothyroid rat. The skin showed thin, folded epidermis,

wrinkled skin and reduced hair follicles as well as mesenchymal cells. We found no such remarkable change in hyperthyroid rat skin.

Histology of Heart Muscle

Significant changes were observed in the histology of heart muscle in the hyperthyroid group when compared with the control group under light microscope (Figure 2A andB). Hyperthyroidism showed hypertrophy of heart muscle.

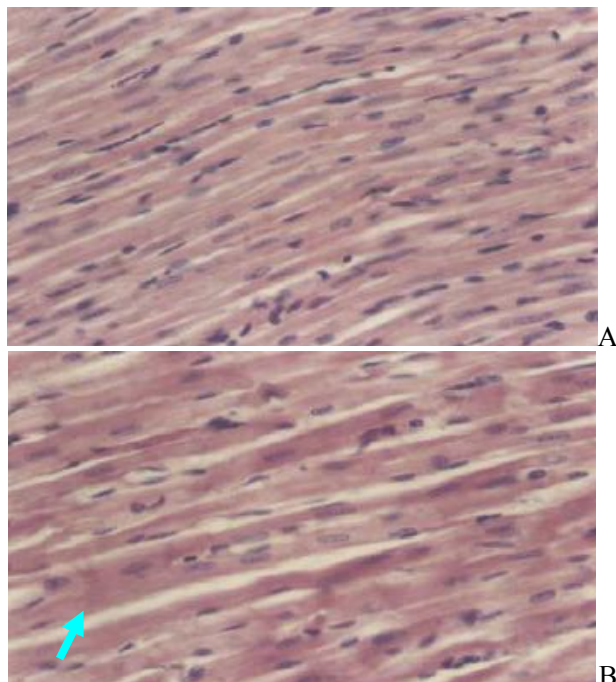


Figure 2. (A, B): (A) Normal rat heart muscle (x 400), (B) Hyperthyroid rat heart muscle (x 400) as indicated by the arrow.

Serum SGOT

Serum SGOT level was significantly elevated (~9.8-fold) in hyperthyroid rats when compared with control rats (Figure 3).

Insulin Tolerance Test (ITT)

In the control rats the blood glucose level returned to the normal level after 120 minutes of insulin injection. Like control rats, in hypothyroid and hyperthyroid rats glucose level significantly decreased after 30 minutes of insulin challenge but the decreased glucose did not returned to basal level after 120 minutes (Figure 4).

Rats	SGOT(U/L)
Control	428.80 $\pm$ 0.40
Hyperthyroid	2529.60 $\pm$ 0.42**

[Values are mean $\pm$ SE from 6 animals in each group].

** $P < 0.001$.

Values are expressed as Mean $\pm$ SE from 6 rats. $P < 0.001$

Figure 3. Serum glutamic oxaloacetate transaminase (SGOT) level (U/L) in control and hyperthyroid rats.

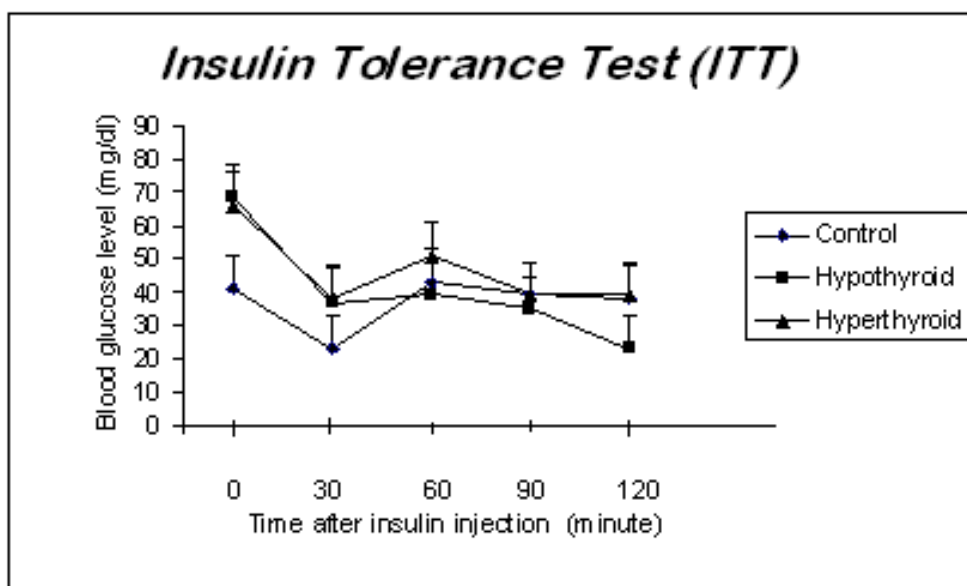


Figure 4. Insulin Tolerance Test (ITT) of control, hypo and hyperthyroid rat.

DISCUSSION

In the present investigation, we used methimazole to induce hypothyroidism and thyroxine to induce hyperthyroidism, as reflected in the serum T3, T4 levels (Pantos *et al.*, 2005; Grofte *et al.*, 1997). We found changes in the histology in cardiac muscles cells after the induction of hyperthyroidism. The size, shape and morphology of cells were significantly changed. The muscle cells also showed significant hypertrophy. Earlier research revealed that thyroid hormones (THs) effects on left ventricular (LV) function and myocyte shape and its remodeling in rats with spontaneously hypertensive heart failure. High dose TH produces signs of increased heart weight, tachycardia (Thomas *et al.*, 2005). Thus, result indicates that thyroid hormones may have direct regulation on cardiac functions. SGOT is normally present in the liver and heart cells and released into blood when the liver or heart is damaged.

We studied the histological changes of skin upon experimentally induced hypo and hyperthyroid conditions in rat. Results showed hypothyroidism altered the general

morphology of skin. The epidermis became folded and hair follicles reduced in number. The result may indicate thyroid hormone may play an important regulatory function in mammalian skin. Our findings also proved the earlier research where hypothyroidism slowed the metabolic rate due to alteration of glandular function (Joshua, 2003).

Thyroid hormone status is also important for glucose homeostasis (Chakrabarti *et al.*, 2007). Impaired glucose tolerance is a frequent complication of hyperthyroidism. This alteration changes both insulin secretion and degradation in humans (Dimitriadis *et al.*, 1985). Impairment in the insulin-induced suppression of glucose production in hyperthyroid patients has been reported (Laville *et al.*, 1984). At low insulin levels, insulin-stimulated glucose disposal is usually unaffected (Muller *et al.*, 1995) whereas it has been reported to be decreased (Dimitriadis *et al.*, 1985), unchanged (Randin *et al.*, 19984) or even increased (Muller *et al.*, 1986; Suthijumroon *et al.*, 1988; Barzilai, 1993) at high insulin levels. In addition, thyroid hormones also blunt the insulin-induced increases in the total distribution volume of the exchanging pool of glucose, possibly by accelerating intracellular glucose degradation (Laville *et al.*, 1984; Chakrabarti *et al.*, 2007). Moreover, thyroid hormones may act as insulin antagonists. Hyperthyroidism induces glucose intolerance in animals and humans. In prone individuals, hyperthyroidism can induce a so-called 'thyroid diabetes'. Thyroxine treatment causes a state of experimental hyperthyroidism and reduces the pancreatic insulin content and glucose-induced insulin secretion.

In our study the blood glucose level of control rats were returned to the basal level after 120 minutes of insulin injection. Unlike control rat, in experimental rat decreased glucose level did not returned to basal level after 120 minutes of insulin challenge. Thus medical efforts should be directed towards prevention of over-challenging the insulin secretory reserve of the endocrine pancreas such as in hyperthyroidism or during exogenous thyroid hormone administration. This could be particularly important in people with a limited endogenous insulin secretory capacity who are most susceptible to the development of 'thyroid diabetes' such as elderly patients with a borderline insulin secretory capacity. This should be kept in mind also when, for therapeutic reasons, thyroid hormone supplementation is considered.

ACKNOWLEDGMENTS

This study was supported by Minor Research Grant from University Grants Commission, New Delhi, India (Sanction: F. No. 33-476/2007 (SR), Dated: 28.02.2008).

REFERENCE

- Barzilai, N; Karnieli, E; Barzilai, D; Cohen, P. Correlation between glucose disposal and amino acids levels in hyperthyroidism. *Horm Metab Res*, 1993. 25, 382-385.
- Chakrabarti, S.; Guria, S.; Samanta, I.; Das, M. Thyroid dysfunction modulates glucoregulatory mechanism in rat. *Indian J. Exp. Biol.*, 2007. 45(6), 549-553.

- Dimitriadis, G; Baker, B; Marsh, H; Mandarino, L; Rizza, R; Bergman, R; Haymond, M; Gerich, J. Effect of thyroid hormone excess on action, secretion, and metabolism of insulin in humans, *Am. J. Physiol.*, 1985. 248, E593-E601.
- Dimitriadis, GD; Richards, SJ; Parry-Billings, M; Leighton, B; Newsholme, EA; Challiss, RA. Beta-adrenoceptor-agonist and insulin actions on glucose metabolism in rat skeletal muscle in different thyroid states. *Biochem J.*, 1991. 278 (2), 587-593.
- Fujita, H. Functional morphology of thyroid. *Int. Rev. Cytol.*, 1988. 113, 145-150.
- Grofte, T; Wolthers, T; Jensen, DS; Moller, N; Jorgensen, JO; Orskov, H; Vilstrup, H. Hepatic amino nitrogen conversion and organ N-contents in hypothyroidism, with thyroxine replacement, and in hyperthyroid rats. *J. Hepatol.*, 1997. 26, 409-416.
- Hoppner, W. and Seitz, HJ. Effect of thyroid hormones on glucokinase gene transcription in rat liver. *J. Biol. Chem.*, 1989. 264(34), 20643-20647.
- Joshua, D; Fraser LM; Ray, S; Holick, M F. Topical Triiodothyronine Stimulates Epidermal Proliferation, Dermal Thickening, and Hair Growth in Mice and Rats. *Thyroid*, 2001. 11(8), 717-724.
- Laville, M; Riou, J P; Bougneres, P F; Canivet, B; Beylot, M; Cohen, R; Serusclat, P; Dumontet, C; Berthezene, F; Mornex, R. Glucose metabolism in experimental hyperthyroidism: intact in vivo sensitivity to insulin with abnormal binding and increased glucose turnover. *J. Clin. Endocrinol. Metab.*, 1984. 58, 960-965.
- Muller, M J; Reynard, C A; Burger, A G; Toffolo, G; Cobelli, C; Ferrannini, E. Kinetic analysis of thyroid hormone action on glucose metabolism in man. *Eur. J. Endocrinol.*, 1995. 132, 413-418.
- Muller, MJ; von, Schutz B; Huhnt, HJ; Zick, R; Mitzkat, HJ; Von, ZMA. Glucoregulatory function of thyroid hormones: interaction with insulin depends on the prevailing glucose concentration. *J. Clin. Endocrinol. Metab.*, 1986. 63, 62-71.
- Pantos, C; Mourouzis, I; Malliopolou, V; Paizis, I; Tzeis, S; Moraitis, P; Sfakianoudis, K; Varonos, DD; Cokkinos, DV. Dronedarone administration prevents body weight gain and increases tolerance of the heart to ischemic stress: a possible involvement of thyroid hormone receptor alpha1. *Thyroid*, 2005. 15, 16-23.
- Parakkal, PF. Mordanting fixation as a means of facilitating the staining of pancreatic cells of mouse. *Stain Technol*, 1961. 36, 33-34.
- Randin, JP; Tappy, L; Scazziga, B; Jequier, E; Felber, JP. Insulin sensitivity and exogenous insulin clearance in Graves' disease. Measurement by the glucose clamp technique and continuous indirect calorimetry. *Diabetes*, 1986. 35, 178-181.
- Reitman, S. and Frankel, SA. colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.*, 1957. 28, 56-63.
- Suthijumroon, A; Toth EL; Crockford, PM; Ryan, EA. Insulin action is altered in hyperthyroidism. *Clin. Invest. Med.*, 1988. 11, 435-440.
- Segal, J.; Masalha, S.; Schwalb, H.; Merin, G.; Borman, JB.; Uretzky, G. Acute effect of thyroid hormone in the rat heart: role of calcium. *Journal of Endocrinology*, 1996. 149, 73-80.
- Thomas, TA; James, A; Kuzman; Brent E; Anderson, MK; Andersen, EHS; Maurice S; Holder, A; Martin G. Thyroid hormones induce unique and potentially beneficial changes in cardiac myocyte shape in hypertensive rats near heart failure. *Am. J. Physiol Heart Circ. Physiol.*, 2005. 288, H2118-H2122.

ONCOTIC CYTOLOGY IN THE PREVENTION OF CERVICAL CANCER

***Melissa Cesar, Luma Rebeca Pedroso, Marcela Cristina Altvater,
Liya Mikami and Kárita Cláudia Freitas-Lidani****

Faculdades Integradas do Brasil – Unibrasil, Curitiba-PR, Brasil.

ABSTRACT

The Oncotic cytology appeared from a study developed by George Papanicolaou, with the objective to analyze the cervical squamous cells in all stages of the menstrual cycle periods, becoming a basic mean of prophylaxis for the cervical cancer. The technique consists of the analysis of the slides with cervical smear and of the detection of cellular alterations with pre-malignant or malignant characteristics. During the cytological analysis fungus, parasites, signals of inflammation, among others alterations can be found. The results are formatted following to Bethesda System, to Report of Papanicolaou or Brazilian Nomenclature for Report of Cervical Pathologies. This article had the goal to introduce a review about the characteristics of the cervical cancer and the techniques that are used to diagnostic it.

Descriptors: Oncotic cytology, Papanicolaou, Cancer of the cervix.

INTRODUCTION

In 90% of the cases of the cervical cancer has the main triggering factor is human Papillomavirus (HPV). Its detection is performed by preventive examination or colposcopy, and according to the Ministry of Health these exams must be performed around 20 years old, because the highest level of incidence of *in situ* carcinoma is age from 20 to 30 years old. According to Incidence Estimates of Cancer in Brazil, in 2006, occurred around 19.260 new cases of cervical cancer [1]. In cases that infection by HPV is positive, the treatment varies from local biopsies to increase the immunity of the local, (in cases of Cervical Intraepithelial Neoplasia I - CIN I), cauterizations (in cases of Cervical Intraepithelial Neoplasia II – CIN II

* Mail address of corresponding author: kari.lidani@gmail.com Telephone number: 55 (41) 3361.4200; Fax number: 55 (41) 3361.4200 Postal address: Konrad Adenauer, 442, Tarumã. CEP: 82820-540 Curitiba (PR)

– with low extension), and even total or partial hysterectomy, external radiotherapy and/or chemotherapy, in the cases of *in situ* and confirmed invasive carcinoma [2].

Great part of developing countries has adopted tracking programs of cervical cancer, whose techniques of prevention started from the work of George Papanicolaou in 1940, and has got good results referring to decrease of death and of the cases of total hysterectomy [3]. However, the technique is still unknown for some Brazilians, even it has been offered for free at units of Unique System of Health (SUS – Sistema Único de Saúde). To make the diagnostic of cervical cancer it's necessary to use the oncotic cytology, technique that analyze microscopically, cervical smears, collected in preventive exam or a biopsy of this tissue [4]. It's analyzed the morphology of the cervical cellular alterations, the microbiological evaluation and detection of inflammation, etc [5, 6].

By the citopathologic evaluation, a simple exam, but extremely detailed, it's possible to prevent the cervical cancer. The purpose of this article is to show a general and clear view of what's cervical cancer and how the oncotic cytology can be used to diagnostic it quick and safely [1,2].

CELLULAR ALTERATION: NORMAL AND PATHOLOGICAL

The uterine cervix is coated, in an orderly manner, by layers of squamous epithelial cells. When they are exposed to some kind of trauma they can suffer progressive intraepithelial transformations, these transformations have the tendency to evolve to a cancerous invasive lesion in a period of 10 to 20 years. Great part of the cases, the evolution of the cervical cancer is slow, going through pre-clinical detectable and curable stages [1].

The stratified epithelia are constantly renewed by physiological factors, however, during the normal process of renewing can occur some structural and functional alterations, as in the nucleus, cytoplasm and organelles, beyond the hypertrophy that is characterized by the increase quantity of the cellular components and functions. It can occur alterations at cellular division classified as hypoplasia that is characterized by the decrease of cellular proliferation, and hyperplasia, defined by the increase of cellular proliferation [7, 8].

Alterations at cellular differentiation re called metaplasia, where the transformation of one type of mature tissue (epithelial or mesenchymal) into another tissue with the same origin occurs as consequence the metaplastic tissue becomes more resistance to aggressions. Another kind of alteration is the dysplasia, characterized by cellular proliferations with decrease or no differentiation; it's classified as a growing and differentiation cellular alteration. Dysplasias are associated to metaplastic tissues or they have its their origin at this tissue, called pre-cancerous lesions; however they are different kinds of cancer, because many of them can stop growing or even regress [6, 7, 8].

In the cellular alterations group, the most concerning are the alterations of cellular growing and cellular differentiation, called neoplasia (from Greek *neo* = new; *plasis* = growing), characterized by autonomous cell proliferation, usually accompanied by lost of differentiation. Neoplasia is divided into benign that usually doesn't cause damage and its evolution is slow, and malign that has fast growing and cause alterations at homeostatic system and can take to death [2,9].

CARCINOGENIC AGENTS AND CARCINOGENESIS

Carcinogenic agents are responsible to cell genome aggressions, with DNA alterations (mutation) or abnormal expression of normal genes. These alterations result of a activation or inactivation of genes that control essential function of the cell, as the proliferation, differentiation, death by apoptosis and genome stability. The accumulation of these mutations in these critical genes results into the progressive loss of the homeostasis and the appearance of malign cellular phenotype. The main agents are divided in chemical such as polycyclic aromatic hydrocarbons, hormones and aromatic amines, physical such as ultraviolet (UV) and ionizing radiation (a and gamma rays) and biological such as papilloma virus (HPV), Epstein-Barr virus and human herpes virus. Among the agents with higher rate of incidence as cervical cancer promoter is the HPV [1, 4, 5].

HPV is associated with many different human diseases, including common and genital warts. It's known that there are at least 80 HPV serotypes in the literature and the number continues to grow. In Brazil the HPV types 16 and 18 are most frequently associated with cervical and anal cancer, 8 other types of HPV are associated with benign genital lesions (HPV 6 and 11). Although it's still not clear how the high-risk types cause cancer, studies indicate that malignant transformation involves the products of viral genes E6 and E7, which may exert their effects interfering in proteins that regulate cell growth. Most of the infected does not develop malign disease, it indicates that only HPV infection is not enough to cause cancer, co-factors such as smoking and immune status are necessary for the cancer genesis [4, 5, 6, 10, 11].

All cells of our body have a mechanism of cellular division control and mutations in the genetic content of cell can overcome these defenses and contribute to cancer development [1]. One of these mechanisms is the apoptosis, programmed death of cell, when essential components are injured or the system control is deregulated. The cell cycle consists of 4 stages. At G1 phase (*gap* 1 = interphase), cell size increases and gets prepared to copy its DNA, the copy (replication) occurs in the next phase, called S (synthesis) and allows the cell to duplicate its chromosomes precisely. After chromosome replication process, phase G2 (*gap* 2) begins, that's when the cell gets prepared to M phase (mitosis) – in which the parent-cell, increased, is divided in half, to produce two daughter-cells with the same number of chromosomes, thus, daughter-cells get in the phase G1 immediately and restart the cell cycle [10, 11].

The development of tumor cells is because of the escape of this mechanism. The protein p53, among many of its function, helps in the beginning of apoptosis and the inactivation of this protein reduces the chance of the genetically injured cells are eliminated, starting a carcinogenic process. Another mechanism of cell division control is a mechanism of counting how many times the cell was divided. In this mechanism the ends of chromosomes (telomeres) mark the number of divisions, and in the appropriate moment initiate senescence and death. Two classes of genes have a main function at cancer development, proto-oncogenes stimulate, while suppressor genes inhibit the process of cell division. When mutation occurs the proto-oncogenes become oncogenes, these genes are carcinogenic and cause excessive cellular multiplication. These mutations induce the proto-oncogene to express in excess its protein that stimulates cellular growing or produce it in a more active mode. The suppressor genes of tumors, otherwise, contribute to the cancer development when they are

inactivated by mutations, whose result is the loss of the function of them, this deprives the cell from crucial controls to prevent the inappropriate growth collectively, these two classes of genes are responsible for uncontrolled proliferation found in malign neoplasia [4, 5, 10, 11].

The stages of carcinogenesis are divided in initiation, promotion and progression, when occurs the exposure to a carcinogenic agent that interacts with the DNA causing a permanent mutation, in which the altered cell can be destroyed by the immune system or remain dormant. Then the trigger of the carcinogenesis by prolonged exposure to a promoter agent occur and the appearance of the characteristics of malignancy, such as the ability to grow in other organs (metastasis), respectively [1].

CIN – CERVICAL INTRAEPITHELIAL NEOPLASIA

Cervical Intraepithelial Neoplasia is characterized by the abnormal growth of epithelial tissue from the cervical surface. It refers to a group or a continuous series of specified changes such as: CIN I, mild dysplasia; CIN II; moderate dysplasia until accentuated dysplasia and CIN III, severe dysplasia until *in situ* carcinoma (cancer located in cervical intraepithelial/surface layer tissue) [4].

The cause is unknown, however, many pre-disposition factors (referred to as risk factors) were identified. Less than 5% of all Pap smear reveal a cervical dysplasia. It may occur in women from 15 years old, and with bigger incidence in women between 25 and 35 years old. The biggest risk is associated to multiple sexual partners, early onset of sexual activity (less than 18 years old), early gestation (less than 16 years old) and a previous history of exposure to sexually transmitted diseases, especially HPV (genital warts), genital herpes, or HIV infection [12].

COLLECTION OF BIOLOGICAL SAMPLE FOR CYTOPATHOLOGIC ANALYSIS

The material for cytopathologic analysis can be achieved by: 1) scrapes from the skin or mucous, such as the cervical, spatulas or brushes for making smears or fixation in liquid medium and special preparation in monolayer. This last one can be analyzed automatically, in computer equipment, or manually in conventional microscope. 2) Secretions. 3) Liquids. 4) Needle aspiration, especially those which use thin needles. In this case, nodular lesions of several organs (thyroid, breast, lymph node, etc.), cystic or solid can be diagnosed with good precision [7, 8].

The achieved cells must be properly fixed. The most used fixation solution is alcohol in different concentrations. In the case of Pap smear tests it's important that the smear is fixed immediately, still wet, ethyl alcohol 95%, the drying before fixing makes the smear unsuitable for the cells examination, when colored by the Pap's method. Furthermore, smears dried before fixations are widely used in hematologic coloration. Secretions rich in mucus or protein may be stored in refrigerator for up to a day before being sent to the laboratory,

because the mucus protects the cells and proteins serve as nutrients. Liquids with low protein and mucus may be kept in the refrigerator for a few hours [8]

The collecting procedure is performed in the ectocervix and endocervix, using Ayres Spatula and a Fields of Peace Brush. After collection, the slide fixation is immediately and it's properly identified. The method of coloration used for the study in histological cuts is applicable to cytological smears. It's clear that in the daily routine, it's used a simple coloration for the preparation that can be examined in the optical microscope. The material fixed in alcohol may be colored with hematoxylin-eosin (HE) or by Papanicolaou technique; so the material is left to get dry on air and can be colored with *Leishman* or *Giemsa*. Both materials allow the implementation of other special techniques of coloration and histochemical and immunochemical reactions (immunoperoxidase technique, for example). The microscopy with phase contrast, with polarized light, with fluorescent light, and even the electron microscope can also be applied, usually at researches [4].

The result of cytological examination is made based on the terms of the morphological diagnosis of diseases and supplemented, when possible, with other data with clinical interest. In the general the pathologist searches for additional information, such as the presence of microorganisms or, in positive cases for cancer, its cytologic type. It's a basic rule that, in principle a radical treatment must not be performed based only on the cytological result exam, except in large interdisciplinary centers, such as in breast pathology, in which the cytologic exam can be decisive when complemented by clinical examination and other secondary exams [14].

OVERVIEW OF THE CYTOLOGICAL ANALYSIS

The oncotic cytology analysis is a diagnostic technique used in the exam of cells from different regions of the organism to determine the cause or the nature of the pathology. The first cytopathological method is dated from XIX century and now is considered a routine method in the diagnosis and prevention of cancer [7]. The cervical screening is an important mean of diagnosis of many diseases, especially malign cancer and its precursor lesions. In this sense, the best example is the Pap smear test for the early detection of cervical cancer. In all countries where programs of mass examination of the population were established and well-conducted, the mortality of cervical cancer has fallen in a remarkable way. In addition to very useful in the diagnosis of neoplastic lesions, the cytological exams are also used to detect of infectious and parasitic agents [12].

The analysis of oncotic cytology has advantages and limitations when compared to histological sample examination. The minor trauma with lower risk of bleeding, the largest surface of the injured tissue sample, the possibility of greater access to tumors located in sensitive areas and well protected, the quick diagnosis, the high rate of detection of malignancy and better relation of cost-effectiveness are the major advantages of oncotic cytology [7]. However, the classification of the type of the tumor, because of the small size of the sample and the detection of the extension and depth of the invasion, are factors that limit the technique. The accuracy of the cytopathologic method depends on several factors, including, the method of the collection of the sample, the suitability of the sample, the examiner's knowledge and experience [7, 8].

Often, the benign diseases may mimic malignant lesions, such as the case of certain immune diseases causing a severe cellular atypia in serous cavities. Without this clinical information, mesothelial cells from ascites fluid may be erroneously interpreted as cancerous cells [8].

CYTOPATHOLOGICAL REPORTS

In the cytological analysis is possible to observe the cellular arrangement (if there are or not cells to be evaluated), size and/or shape variations (changes of volume or in cell division), cytoplasm (if it is present in the cells and contains organelles), core (one or more nuclei), the extracellular material and/or the background of the smear (microorganisms, parasites, bleeding, inflammation) [13].

In 1988, pathologists and gynecologists met in Bethesda (Maryland – USA) and made the Bethesda System of Reports, in addition to this, there is also the World Health Organization (WHO), Cervical Intraepithelial Neoplasia (CIN) and the Brazilian nomenclature for cervical reports. Many methods are used for the report with the cervical results. Papanicolaou created a method of numeric classification ranged from Class I, absence of abnormal cells, to Class V, for conclusive evidence of cancer [15].

Each cytology report must include elements such as, an assertion about the adequacy of the sample for the diagnostic evaluation, a general classification such as “abnormality of epithelial cells” and the interpretation and the analysis of results [8, 15].

CONCLUSION

Cervical cancer mainly affects the most vulnerable social groups, where the major difficulties to the access to care for the early detection and treatment of the disease, such as the deficiency of public health services, cultural issues, fear and prejudice of the partners are concentrated.

The best way of prophylaxis is the preventive examination, colposcopy and if necessary the cytological analysis, which show a great efficiency in the morphological and microbiological classification of the cervical smears, contributing to the appropriate treatment of pre-neoplastic lesions and tumors.

For the effectively prevention of cervical cancer it's necessary the divulgation in a wide and simple way about its risk factors and how the prevention is done, beyond a greater incentive for the preventive exam.

REFERENCES

- [1] Barbosa, A. *Câncer, direito e cidadania*. 12^a ed. São Paulo: ARX, 2007.
- [2] Buzaid, AC. *Manual Prático de Oncologia Clínica do Hospital Sírio-Libanês*. Edição de Antonio Carlos Buzaid. 2^a. ed. Rio de Janeiro: Reichmann and Affonso, 2004.

- [3] Coelho, FRG. *Cancer : manual de orientação para pacientes e interessados*. 2^a. ed. São Paulo: Robe, 2000.
- [4] Faria, JL. *Patologia geral : fundamentos das doenças, com aplicações clínicas*. 4^a. ed. Rio de Janeiro: Guanabara Koogan, 2003.
- [5] Montenegro, MR; Franco, M. *Montenegro e Franco patologia : processos gerais*. 4^a. ed. São Paulo: Atheneu, 2006.
- [6] Brasil. Ministério da Saúde. Secretaria de Vigilância Sanitária. Programa Nacional de DST E AIDS. *Manual de controle doenças sexualmente transmissíveis - DST*. Brasília: Ministério da Saúde, 2006.
- [7] Michalany, J. *Anatomia patológica: prática e propedêutica*. 1^a. ed. São Paulo: Lemos, 2005.
- [8] Rubin, E. *Patologia: bases clinicopatológicas da medicina*. 4^a. ed. Rio de Janeiro: Guanabara Koogan, 2006.
- [9] Brasileiro Filho, G. *Bogliolo patologia geral*. 3^a. ed. Rio de Janeiro: Guanabara Koogan, 2004.
- [10] Carneiro, J; Junqueira, LC. *Biologia celular e Molecular*. 7^a. ed. Rio de Janeiro: Guanabara Koogan, 2000.
- [11] Alberts, B. *Fundamentos da biologia celular: uma introdução à biologia molecular da célula*. Colaboração de Dennis Bray et al. 1^a. ed. Porto Alegre: Artmed, 2002.
- [12] Brasil. Ministério da Saúde Prevenção do câncer do colo do útero. *Manual técnico*. Brasília, 2002.
- [13] Takahashi, M. *Atlas colorido de citologia oncótica*. 2^o ed. São Paulo: Mande Ltda, 1982.
- [14] Brasileiro F. G. *Bogliolo Patologia*. 6^a. ed. Rio de Janeiro: Guanabara Koogan, 2000.
- [15] Gomes, FMP. Nomenclatura brasileira para laudos cervicais e condutas preconizadas recomendações para profissionais de saúde. *Rev. Bras. Ginecol. Obstet.* vol.28, n^o. 8, Rio de Janeiro, 2006.

CYTOTOXIC RESPONSE AND NONSELF PHAGOCYTOSIS AS INNATE IMMUNITY OF MOLLUSC UNDER ARSENIC EXPOSURE

Sudipta Chakraborty¹ Mitali Ray² and Sajal Ray^{2*}

¹Post Graduate Department of Zoology,

Parasitology and Medical Entomology Laboratory,

Darjeeling Government College, Darjeeling-734101, West Bengal, India

²Department of Zoology, Aquatic Toxicology Laboratory, University of Calcutta,
35 Ballygunge Circular Road, Kolkata – 700019, West Bengal, India

ABSTRACT

Haemocyte mediated non-self phagocytosis and generation of nitric oxide (NO) as reactive nitrogen intermediate were examined to investigate the acceptability of the parameters as biomarkers of sodium arsenite induced stress on *Lamellidens marginalis* (Mollusca; Bivalvia; Eulamellibranchiata), at sublethal concentrations. The animals suffered a remarkable immune compromise by the natural toxicant. Partial recovery was observed in the phagocytic potential and NO generation by the haemocytes of the animals over a period of thirty days. Quantitation of phagocytic efficiency and intrahaemocyte NO generation suggests reliability of the parameters as cellular biomarkers of sodium arsenite induced stress on freshwater molluscs.

Keywords: *Lamellidens marginalis*; haemocyte; sodium arsenite; phagocytosis; nitric oxide; biomarker.

INTRODUCTION

The filter feeding bivalve *Lamellidens marginalis* forms a common dietary item for the rural mass of the eastern India and a popular poultry feed (Chakraborty *et al*, 2008). The species is harvested indiscriminately from their natural habitats and no scientific culture system for the rearing and breeding of the species has been reported. Occasionally, natural

* Corresponding author: Department of Zoology, Aquatic Toxicology Laboratory, University of Calcutta, 35 Ballygunge Circular Road, Kolkata – 700019, West Bengal, India. E-mail: raysnailmail@rediffmail.com Fax: 913324614849

pearl is found in the species which highlights its commercial potentiality. The natural habitats of *L. marginalis* are threatened by the risk of contamination by arsenic, a major water contaminant of the Gangetic basin of India and Bangladesh (Acharyya *et al.*, 1999) and it is reported that biochemical leaching of the earth crust sediment cause mobilization of the poison in water (Islam *et al.*, 2004). Extensive use of arsenic contaminated groundwater for agriculture is in practice in India and the agricultural runoffs contaminate the field adjacent freshwater bodies (Islam *et al.*, 2000) which form the natural habitat of *L. marginalis* and many other organisms. The stratification of lakes in summer cause a 10-20% increase in arsenic concentration in water (Aggett and O'Brien 1985) and in the water of the interstitial lake, 90% arsenic exists as arsenite, a highly toxic trivalent form of arsenic (Aggett and Kriegmann 1988). Although well documented in mammalian systems, the effects of arsenic toxicity on freshwater bivalve in Indian subcontinent is limited (Chakraborty *et al.*, 2009).

The haemocytes are the circulating cells of the bivalves that act as the major immune effector cells (Cheng 1977, Adema *et al.*, 1991b). Haemocyte mediated non-self phagocytosis greatly strengthens the immunity in the bivalves (Lopez *et al.*, 1997). Nitric oxide (NO) is produced as a reactive nitrogen intermediate during conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (Ischiropoulos *et al.*, 1992). NO has the ability to kill foreign micro-organisms by combining with superoxide (O_2^-) to form peroxynitrite, a strong bactericidal agent (Arumugan *et al.*, 2000). In this study, the phagocytic efficiency and the rate NO activity of the haemocytes of *L. marginalis* was estimated under the *in vitro* challenge of human red blood corpuscles (HRBC) by exposing the animals to sublethal concentrations of sodium arsenite in controlled laboratory conditions so as to authenticate the immune parameters as biomarkers of aquatic toxicity.

MATERIALS AND METHODS

Collection and Treatment of Animals

Fresh specimens of *L. marginalis* (average size of 7.002 x 4.135 cm) were collected from some selected freshwater ponds of the district of 24-Parganas (South) of West Bengal in India. The collected animals were transported to the laboratory and acclimated for 5-7 days in well-aerated tanks where they were reared and maintained according to Raut (1991). Aqueous solutions of sodium arsenite (E. Merck, Germany; 99% pure; CAS number 7784-46-5) were prepared in Borosilicate glass containers. The sublethal concentrations of sodium arsenite were 1, 2, 3, 4 and 5 ppm and the pH of the solutions was maintained at 7.2. Each experimental set consisted of 10 animals of the identical size and they were exposed to a volume of 5 l of sodium arsenite solution. For control, a set of animals were kept in arsenic free analytical grade water. The experiments were carried out in static water environment and fresh solutions of sodium arsenite were replenished every 12 h.

For the recovery assay, sets of 10 animals were first exposed to solutions of sodium arsenite. After exposure for 24, 48, 72, 96 h and 30 days, the animals were shifted to arsenic free analytical grade water to examine possible restoration activity. The ambient temperature of the water was maintained between 24 -26 °C.

Collection of Haemolymph

Aseptic collection of haemolymph was done from the heart of the animals after Brousseau *et al.*, (1999) and was stored in prechilled glass vials. A portion of the fresh haemolymph was smeared on clean and sterilized glass slides in moist chamber so as to get a haemocyte monolayer on the glass surface. The haemocytes were allowed to settle for 15 min at room temperature. The viability of the haemocytes of *L. marginalis* in all experimental variants was tested with 2% Trypan blue for 10 min following dye exclusion principle and the average viability of haemocytes ranged between 96.30 % to 92.50% in all the experimental variables.

Preparation of HRBC Suspension

Human blood samples (expired stock) were collected from a local hospital of South 24-Pargana, West Bengal. The HRBC was washed in PBS (pH 7.2, supplemented with 0.01 M EDTA and 1% glucose) by centrifugation at 650 x g for 10 min. The washed HRBC cell fractions were then resuspended at a concentration of 10^7 cells ml^{-1} . The work was designed in accordance with the guidelines of the institutional (University of Calcutta) norms of animal handling and care.

Phagocytosis

The phagocytic efficiency of the haemocytes was examined by challenging them with HRBC suspension *in vitro* over slide. To the adherent monolayer of haemocytes, 0.1 μl of HRBC (1×10^7 cells ml^{-1}) was added and incubated at 37°C in a humid chamber for 3 hours. After incubation, the monolayer was washed with sterile snail saline (5mM HEPES, 3.7mM NaOH, 36 mM NaCl, 2 mM KCl, 2mM MgCl_2 , $2\text{H}_2\text{O}$, 4mM CaCl_2 , $2\text{H}_2\text{O}$; pH 7.8) (Adema *et al.*, 1991a), stained with Giemsa's stain and observed under a light microscope (Axiostar Plus, Zeiss). A negative control for the assay was set using 2% sodium azide, a known phagocytic inhibitor. More than 250 fields were examined for each slide and following data were accounted: number of phagocytic haemocyte, total number haemocyte and number of HRBC particles engulfed by each haemocyte. The data were calculated and represented in terms of phagocytic index (PI), where, $\text{PI} = (\text{particles/cell} \times \text{phagocytic cells} / 100)$ (Elsner *et al.*, 2004). The entire experiment was repeated five times.

Estimation of NO Generation

The generation of NO was measured as the amount of the nitrite released from the haemocytes with Griess reagent after Green *et al.*, (1982) with minor modifications. The concentration of haemocytes was adjusted to 10^6 cells ml^{-1} . For the assay of estimation of NO generation during phagocytosis, suspension of 10^6 haemocytes ml^{-1} was challenged with HRBC at an optimum ratio of 1:10; the cell suspension was incubated at 37°C for 3h in a

humid chamber. One ml of the HRBC challenged haemocyte suspension was incubated with equal volume of Griess reagent (1% Sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% orthophosphoric acid) at 37°C for 30 min in a humid chamber. The absorbance were spectrophotometrically (CECIL- CE 4002) recorded at 550nm against a standard blank. A standard curve of sodium nitrite was used to determine generation of NO. The entire experiment was repeated five times.

Statistical Analysis

The statistical data analysis was carried out using Student's t-test. Differences were considered significant at $P < 0.05$, $P < 0.01$, $P < 0.001$. Data was presented as the mean $\pm$ standard error (S.E.).

RESULTS

Inhibition of Phagocytic Response

The phagocytic response of the haemocytes (Figure 1) challenged with HRBC was suppressed upon exposure of the animals to 1, 2, 3, 4 and 5 ppm sodium arsenite as revealed from the decline in PI. Toxicity of arsenic on haemocyte was maximum under an exposure of 30 days. The PI of the haemocytes exhibited a dose dependent suppression(Figure 2).

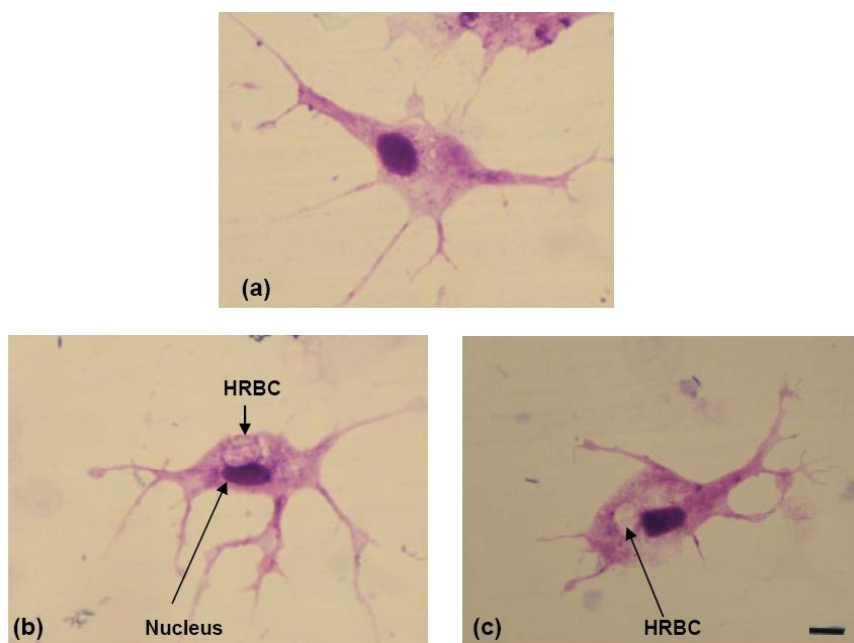


Figure 1. Haemocyte of *L. marginalis* (a) a spreading haemocyte, (b) and (c) haemocytes with engulfed HRBC (bar =5 μ m, magnification 1000x).

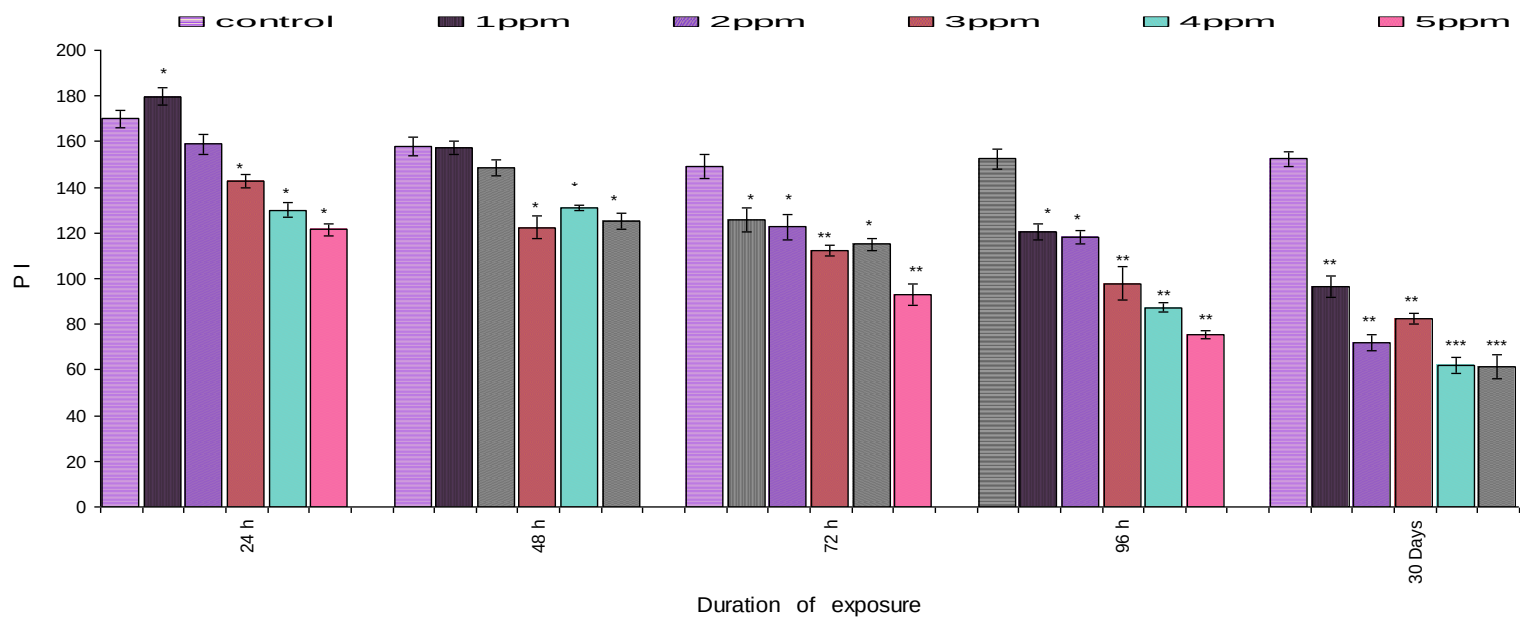


Figure 2. Phagocytic Index (PI) of haemocyte of *L. marginalis* after exposure to 1, 2, 3, 4, 5 ppm of sodium arsenite for 24, 48, 72, 96 h and 30 days under the challenge of HRBC. Data presented as mean $\pm$ SE. (n=5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

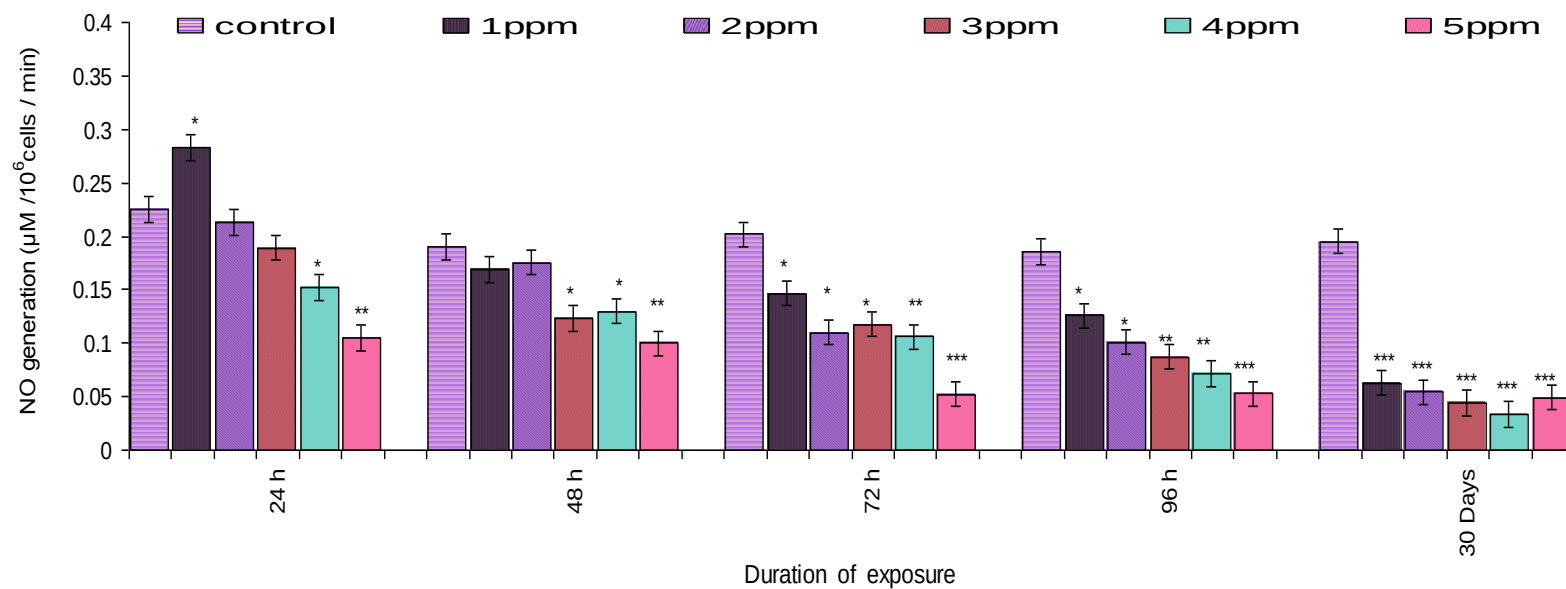


Figure 3. Intrahepatic NO generation of *L. marginalis* after exposure to 1,2,3,4,5 ppm of sodium arsenite for 24, 48, 72, 96 h and 30 days under the challenge of HRBC. Data presented as mean $\pm$ S.E. (n=5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Inhibition in NO Generation

At lower concentrations of sodium arsenite, the generation of NO in haemocytes exposed to HRBC were elevated while the exposure to higher concentrations of toxin resulted a steady decrease in generation of intrahaemocyte NO although mild hormesis was expressed at certain toxin concentrations (Figure 3). Long term exposure to sodium arsenite for 30 days resulted in acute decrease in intrahaemocyte NO generation even under the challenge of HRBC (Figure 3).

Inhibition in Restoration of Phagocytosis and NO Generation

Partial restoration in PI of the haemocytes was observed in toxin free water (Figure 4). In the long term exposure experiments, trend of recovery was identical with other sets and full recovery of phagocytic efficacy was not achieved. Complete restoration in the generation of NO by the HRBC challenged haemocytes of the animals was recorded in the animals exposed to low concentrations of sodium arsenite for 72h (Figure 5). However, recovery was partial for the animals maintained for 30 days in arsenic free water and they generated lower level of intrahaemocyte NO under challenge of HRBC.

DISCUSSION

Phagocytosis is a very important immunological parameter and an useful biomarker of aquatic pollution (Oliver *et al*, 1999). The inhibitory effect of sodium arsenite on the phagocytic efficiency of the haemocytes of *L. marginalis* at varied sublethal exposures was reflected from the present study. The effect of the natural toxin in all the studied concentrations was alarming over persistent exposures. The efforts to restore the normal parameters proved ineffective even after 30 days. The phenomenon signifies a major immune compromise in the bivalve.

In the animal world, NO mediated pathogen killing and superoxide remediation has earned recognition as an effective immune strategy (Bogdan, 2001). On one hand, the toxin suppressed the NO activity while on the other, its residual toxic effect deterred the post-treated animals to restore the normal activity. The NO activity was also found to be down regulated by sodium arsenite exposure when the treated haemocytes were challenged with HRBC. Although a hormetic effect of sodium arsenite on the NO generation was recorded, such stimulation in immunological functions is common under the induction of low concentrations of metallic toxins (Cheng and Sullivan, 1984; Barnier, 1995). The trend of immune suppression persists with further increase in toxin concentration on persistent exposure. Since NO plays vital roles in scavenging O_2^- radicals and production of bactericidal peroxynitrite molecules, its low production might affect the animal in either/both ways: (i) unscavenged O_2^- might impose severe damage to the conformity of the internal cell and tissue structures of the animal, (ii) it might make the animal vulnerable to opportunistic parasitic/microbial attack.

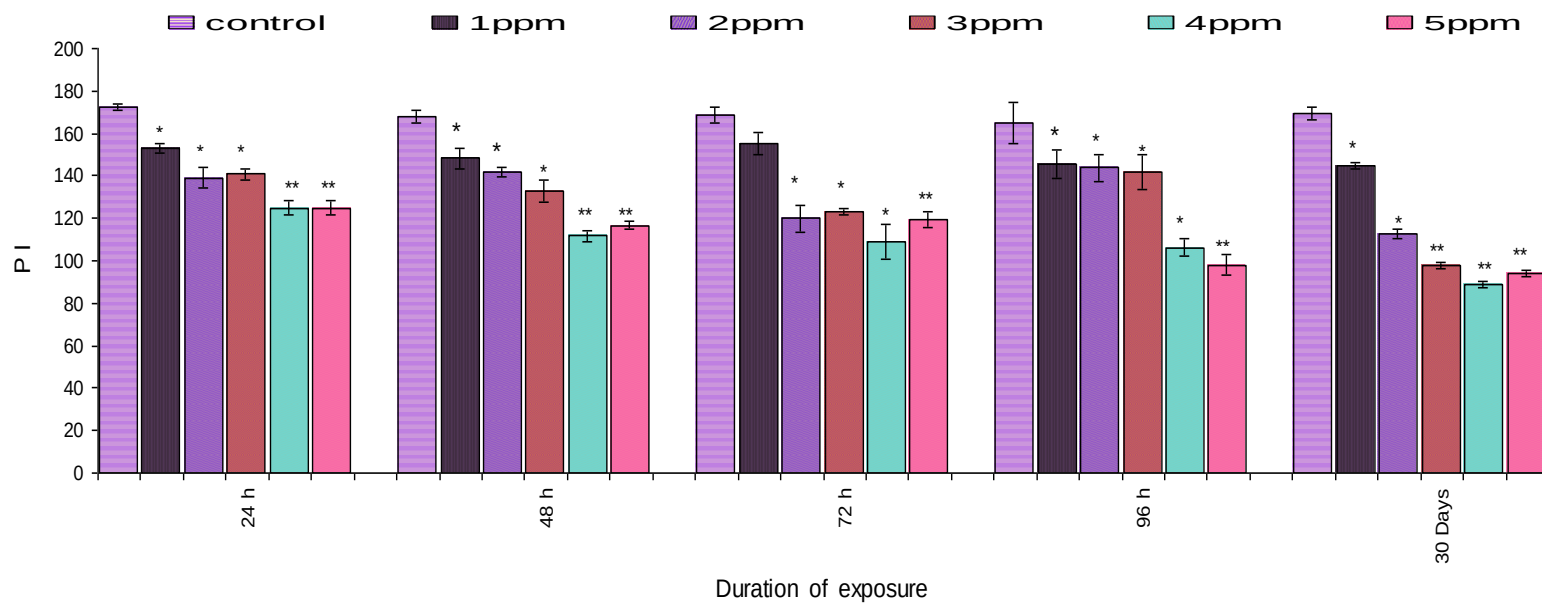


Figure 4. Restoration in phagocytic index (PI) of haemocytes of post-treated *L. marginalis* maintained in arsenic free water for 24, 48, 72, 96 h and 30 days under the challenge of HRBC. Data presented as mean $\pm$ S.E. (n=5). *P < 0.05, **P < 0.01.

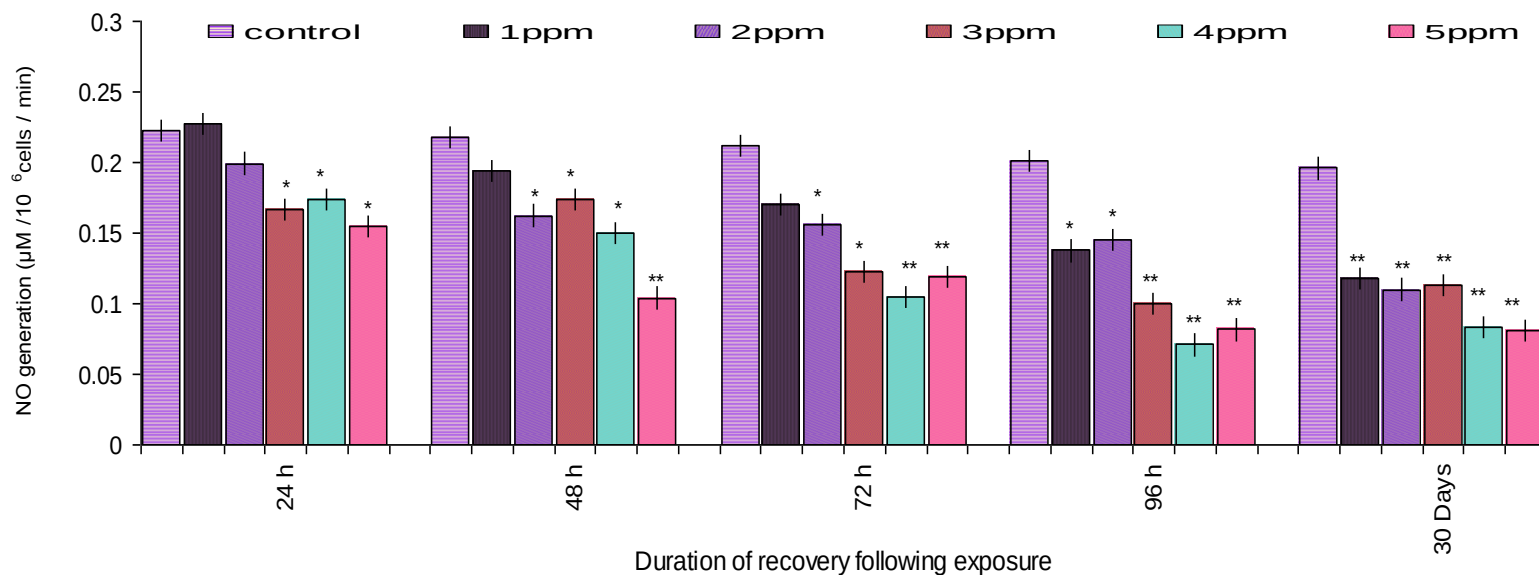


Figure 5. Restoration in NO activity of haemocytes of post-treated *L. marginalis* maintained in arsenic free water for 24, 48, 72, 96 h and 30 days under the challenge of HRBC. Data presented as mean $\pm$ S.E. (n=5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Sublethal toxicity has been reported to affect a population by reducing its fitness thus increasing its susceptibility to higher rate of parasitism and predation (Oliver and Fisher, 1999, Fournier *et al*, 2000). Even if a toxin retards the developmental event rather killing the members of some population, the impact of such event on the ecology probably remains even more far reaching.

Innate immune biomarker like phagocytosis is an useful *in situ* tool for investigation because this assay appears to be effective in field condition and it does not require sensitization of the animals (Fournier *et al*, 2000). The present study confirms that phagocytosis of non-self particles and generation of intrahaemocyte NO bears high credibility to be selected as effective immunological biomarkers for assessing arsenic induced stress due to their dose responsiveness to the toxin in the studied animal.

L. marginalis is a widely distributed aquatic bivalve of India and it exhibits sensitivity to diverse forms of xenobiotics (Chakraborty *et al*, 2008). Aquatic ecosystem of Indian subcontinent supports a wide range of biodiversity which is under threat of environmental contamination. The present study was aimed to establish a dependable as well as an inexpensive biomarker for rapid and accurate screening of the health of the aquatic ecosystem of India. The assays bear high practicability to be chosen as biomarkers of aquatic contamination and for the assessment of xenobiotic induced stress in the aquatic molluscs.

ACKNOWLEDGMENT

Authors thankfully acknowledge the departmental instrumental facility and fund received through DST FIST and UGC SAP DRS, Government of India.

REFERENCES

- Acharyya, S. K.; Chakraborty, P.; Lahiri, S.; Raymahashay, B. C.; Guha, S. and Bhowmik, A. 1999. Arsenic poisoning in the Ganges delta. *Nature*, 401 (6753): 545-550.
- Adema, C. M.; Van Deutekom-Mulder, E. C.; van der Knapp, W. P. W.; Meuleman, E. A. and Sminia, T. 1991a. Generation of oxygen radicals in hemocytes of the *Lymnaea stagnalis* in relation to the rate of phagocytosis. *Dev. Comp. Immunol.*, 15: 17 - 26.
- Adema, C. M.; Van Der Knaap, W. P. W. and Sminia, T. 1991b. Molluscan hemocyte mediated cytotoxicity : The role of reacting oxygen intermediates. *Rev. Aqua. Sci.*, 4: 201-223.
- Aggett, J. and O'brien, G. A. 1985. Detailed model for mobility of arsenic in Lacustrine sediments based on measurements in Lake Ohakuri. *Env. Sc. Tech.*, 19(3): 231-238.
- Aggett, J. and Kriegman, M.R. 1988. The extent of formation of arsenic (III) in sediment interstitial waters and its release to hypolimnetic waters in Lake Ohakuri. *Water Res.*, 22 (4): 407-411.
- Arumugan, M. ; Romestand, B. and Torreilles, J. 2000. Nitrite released in hemocytes from *Mytilus galloprovincialis*, *Cassostrea gigas* and *Ruditapes decussates* upon stimulation with phorbol myristate acetate. *Aqua. Liv. Res.*, 13:173-177.

- Barnier, J.; Brousseau, P.; Krzystyniak, K.; Tryphonas, H. and Fournier M. 1995. Immunotoxicity of heavy metals in relation to Great Lakes. *Env. Health Pros.*, 103 : 23-34.
- Bogdan, C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* 2 (10): 907-916.
- Brousseau, P.; Payette, Y.; Tryphonas, H.; Blakley, B.; Boernaus, H.; Flipo, D. and Fournier, M. 1999. Manual of Immunological Methods, CRS Press, Boca Raton, FL.
- Chakraborty, S.; Ray, and M. Ray, S. 2008. Sodium arsenite induced alteration of hemocyte density of *Lamellidens marginalis* - an edible mollusk from India. *Clean- Soil, Air, Water*, 36(2), 195-200.
- Chakraborty, S.; Ray, and M. Ray, S. 2009. Nuclear morphology and lysosomal stability of molluscan hemocytes as possible biomarkers of arsenic toxicity. *Clean- Soil, Air, Water*, 37(10), 769-775.
- Cheng, T. C. 1977. Biochemical and ultrastructural evidence for the double role of phagocytosis in molluscs: defense and nutrition. *Comp. Pathobiol.*, 3: 21-30.
- Cheng, T. C. and Sullivan, J. T. 1984. Effects of heavy metals on phagocytosis by mollusca hemocytes. *Mar. Env. Res.*, 14:305-315.
- Elssner, A.; Carter, J. E.; Yunger, T. M. and Wewers, M. D. 2004. HIV-1 infection does not impair human alveolar macrophage phagocytic function unless combined with cigarette smoking. *Chest*, 125:1071-1076.
- Fournier, M.; Cyr, D. ; Blakley, B.; Boermans, H. and Brousseau, P. 2000. Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics. *Am. Zoo.* 40: 412-420.
- Green, L .C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S. and Tannenbaum, S. 1982. Analysis of nitrate, nitrite and (N¹⁵) nitrate in biological fluids. *Anal. Biochem.* 126:131-138.
- Ischiropoulos, H.; Zhu, L. and Beckman, J.S. 1992. Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* 298: 446-451.
- Islam, M. R.; Lahermo, P.; Salminen, R.; Rojstaczer, S. and Peuraniemi, V. 2000. Lake and reservoir water quality affected by metals leaching from tropical soils, Bangladesh. *Env. Geol.*, 39 (10): 1083 – 1089.
- Lopez, C.; Carballal, M.J.; Azevedo, C. and Vallalba, A. 1997. Morphological characterization of the hemocytes of the clam *Ruditapes decussates* (Mollusca: Bivalvia). *J. Inv. Pathol.*, 69: 51-57.
- Oliver, L. M. and Fisher, W. S. 1999. Appraisal of prospective bivalve immunomarkers. *Biomarkers* 4(10): 510-530.
- Raut S K. 1991. Laboratory rearing of medically and economically important molluscs. In: M.S. Jairajpuri, editor. Snails, flukes and man. *Zoogical Survey of India Publication*. p 79- 83.

SPAWNING OF THE ENDANGERED KILLIFISH, *APHANIUS DISAR DISPAR* (RÜPPEL, 1829), UNDER LABORATORY CODITIONS

*** El Amin Mohamed Suliman, Ali S. Al-Akel, Hmoud F. Alkhem Al-Balawi, Fahad Al-Misned and Zubair Ahmad**

* Department of Zoology. College of science,
King Saud University, P. O. Box 2455, Riyadh –11451, Saudi Arabia

ABSTRACT

Spawning of the killifish, *Aphanius dispar dispar*, have been studied in freshwater under controlled laboratory conditions. Two breeding systems, with cages and without cages, were used to study the fish spawning. Five replicates for each system were used. One male and two females were stocked in each tank for ten weeks. The total production of eggs in the tanks with cage system was 144 eggs and the average egg production per tank per week was 2.88 eggs. 59 fries were hatched and the hatchability rate was 41%. No eggs were found in the tanks without cages probably because of predation by their parents. There was a highly significant ($P < 0.0001$) difference between the two systems in egg production. It was also observed that the eggs were infected with fungus in the breeding and incubation tanks which reduced the eggs production and hatching rate, and caused fry mortality. The present study revealed that the tanks with cage system was the best method to prevent predation of fish on eggs and fry, and to increase fry production. The Ultra Violet (UV) system is recommended to disinfect water of breeding tanks and incubation jars to eliminate fungal infection of eggs. This will help in mass production of *A. dispar dispar* eggs and fry for restocking of freshwater bodies for biological conservation of the fish to be used for biological control of mosquito larvae and ornamental purpose.

Keywords: Spawning, Killifish, *Aphanius dispar* , Biological control, Fungal infection, Saudi Arabia

INTRODUCTION

Aphanius dispar dispar (Teleostei, Cyprinodontidae) is one of the dwarf native fishes to Saudi Arabia occupying both fresh and marine waters. *A. dispar dispar* is a euryhaline fish found in a wide range of salinities, "from fresh water to 1500‰ (175 ppt) in the dead sea springs" (Plaut, 2000). Lotan (1969, 1971); Lotan and Skadhauge (1972); Skadhauge and Lotan

(1974) and Fouda (2005) Stated that *A. dispar dispar* can sustain their osmotic pressure and ionic concentration when adapted to salinities 16-105 ppt. According to EPAA, (2003): " *A. dispar dispar* is widely distributed along the east cost of the Mediterranean , coastal region of Bahrain, Djibouti, Egypt, Islamic republic of Iran , Iraq, Northern Somalia, Sudan and the Arabian Peninsula (Oman, Saudi Arabia, Yemen) ,up to Pakistan and the west coast of India where , historically , it is well known for its larvivorous efficiency". *A. dispar dispar* is also found in oasis pools with hypersaline to freshwater. It can breed throughout the year with a peak in the period between April and June and well adapted to environmental conditions. It can be used for biological control of mosquito larvae in areas to which it has well adapted (Haas, 1982; Alkahem and Behnke, 1983 and Krupp, 1983 , 1988). *A. dispar dispar* spawn on roots of Hyacinth and other rooting plants in the subtropical area at temperatures ranging from 16 to 26°C (Froese and Pauly, 2009). Al-Akel et. al. (1987) gave a good account on the selective feeding behavior of *A. dispar* in freshwaters of Saudi Arabia; they reported that the fish has high selective feeding capability for food. Frekel and Goren (1997) studied the environmental factors that affect spawning of *A. dispar* especially temperature and the photoperiod.

The population of *A. dispar dispar* is declining as a result of several factors and now the fish is endangered. There is a great need to breed this species for restocking the natural freshwater bodies to save it from extinction as well as to ensure the availability of the fish for the control of mosquito larvae. The declining factors of *A. dspar dispar* in the freshwater environment of Saudi Arabia are summarized by AL-Kahem-Albalawi, et. al. (2008) as a combination of factors affecting the different stages of their life cycle. They include the reduced availability of food, habitat degradation, chemical pollutions , introduction of exotic species, and fish exploitation which lead to declining of such native fish's abundance. Al-Kahem (personal observation), stated that " the population of this species in Al Hasa irrigation canals are declining and in Al Khurj the fish are completely lost due to drying of the water bodies. Several authors stated that *A. dispar dispar* is endangered fish species and there is a need to save their population from extinction. Protecting the fish in general will help in protecting the aquatic biodiversity and biological conservation of fishes of the country (Al-Kahem-Albalawi, et . al. ,2008).

A successful attempts to breed *A. dispar dispar* under controlled conditions for restocking the natural freshwaters will prevent further declining of the fish in freshwater environment, and make them available for biological control and as aquarium fish for the economic and recreational importance.

In this study, two breeding systems (a cage system and a system without cage) were used to evaluate their reproductive performance.

MATERIALS AND METHODS

The killifish , *Aphanius dispar dispar*, (total length 3.2 - 5.3 mm and body weight 1.0 - 2.2 gm) were obtained from irrigation canals of Al-Hasa province and brought to the zoology department laboratory, faculty of science, King Saud university in Riyadh to study the spawning. The fish were brought in May, 2009 and acclimatized to laboratory conditions for one month. The experiment started on 1 /7 2009 lasted for ten weeks on 10/9/ 2009. One

mature male and two females were stocked in each tank. Two systems were selected for studying the spawning of the fish. The first system was a suspended mesh cage and the other system without cage. Each system has a set of five cylindrical tanks (25L capacity). The fish were fed with aquarium flakes - aquafire (38% protein). The photoperiod extended to 12 hours a day. Temperature is adjusted to 23-24°C by aquarium heaters. Tanks were aerated by continuous supply of fresh air. The tank water changed weekly and at the same time eggs were collected. Eggs were examined under the microscope for infection and their general conditions. The infected eggs were identified and photographed under light – microscope (fig, 2). A record was maintained for the number of eggs per week per tank. The eggs were incubated in jars (1000 ml) until hatching. Incubators were aerated up to oxygen saturation (7.5-8.0 mg/l) and temperature was adjusted to 23-24°C. The hatched fry were counted. Hatchability was determined. Then fry were transferred to other jars for feeding with powder feed prepared from aquarium flakes of the brooder fish (38 % protein).

Statistical analysis of T-Test , Two pairs was performed to evaluate the two systems of cage and without cage for breeding performance of *A. dispar dispar*.

RESULTS

After the adjustment of temperature to 23-24 °C and the photoperiod to 12: 12 hrs (day and night) the fish started to spawn in the breeding tanks. Breeding of *Aphanius dispar dispar* occurs in the caged system only. No eggs were found in tanks without cages, although the same conditions were maintained for the two systems during the period of the experiment. Data for the eggs produced during the period of study are summarized in Table (1) and figure (1). The total number of eggs produced were 144 and the average number per tank per week were 2.88 eggs. The hatched fry number was 59 and the egg hatching rate was calculated as 41% of the incubated eggs. When fry are stocked together. It was observed that larger fry feed on the small fry and show cannibalistic behavior.

The T-test for performance of the two systems (with cages and without cages) shows a highly significant ($p < 0.005$) difference between the cage and without cage systems (table, 2).

The new eggs were transparent, gradually become more opaque towards development of the eggs. At the later stages of development and before egg hatching fry are observed moving inside the eggs. Egg hatched in the hatching jars and the yolk sack is absorbed after 2 days from hatching. The fry now supplied the powder food. Eggs and fry of *A. dispar dispar* are heavier than water: they always settled down on the bottom of the tank.

The some eggs examined under the microscope were found infected with fungus which kills the embryos and (or) stop the development of the eggs (figure 2).

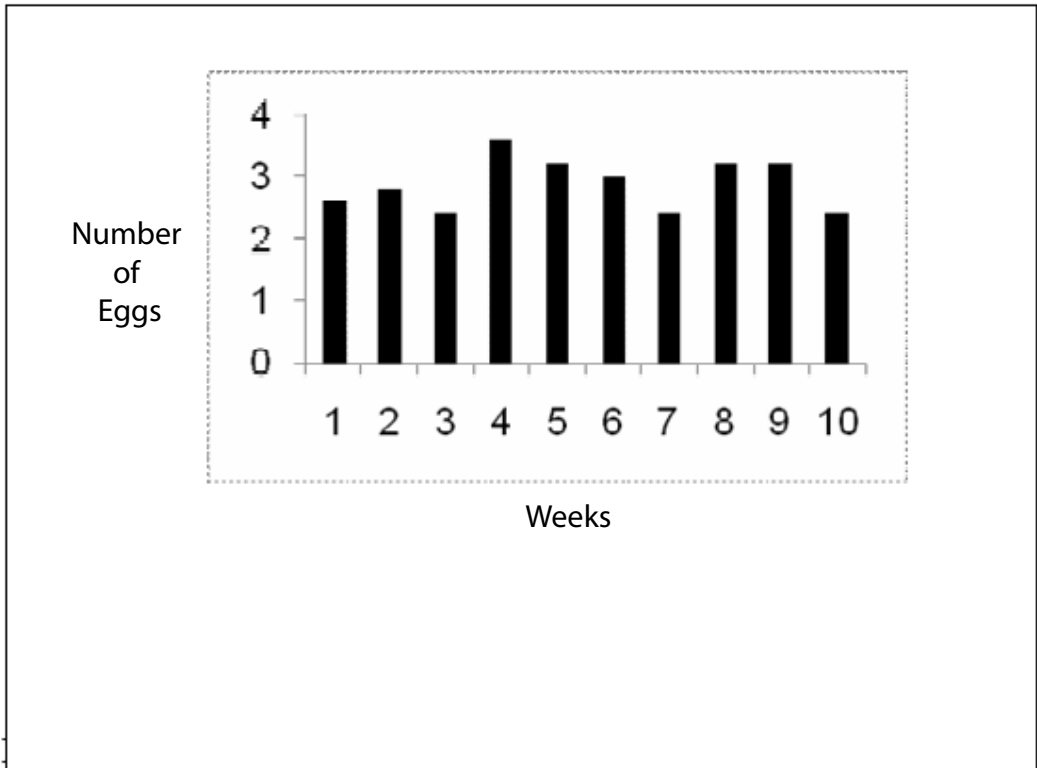


Figure 1. Average number of *Aphanis dispar* eggs /tank/week.

Table 1. *Aphanis dispar* eggs collected from the tank cag system during the experimental period (10 weeks)

Weeks of sampling	Tank-1	Tank-2	TanK-3	TanK-4	TanK-5	total	average
	Egg Number/week						
1	1	5	0	4	3	13	2.6
2	2	5	0	7	0	14	2.8
3	3	4	2	0	3	10	2.4
4	6	3	0	3	6	18	3.6
5	2	0	4	5	5	16	3.2
6	0	3	5	2	5	15	3.0
7	3	3	1	2	3	12	2.4
8	2	0	5	4	5	16	3.2
9	5	3	6	0	2	16	3.2
10	2	1	3	6	0	12	2.4
Total						144	2.88

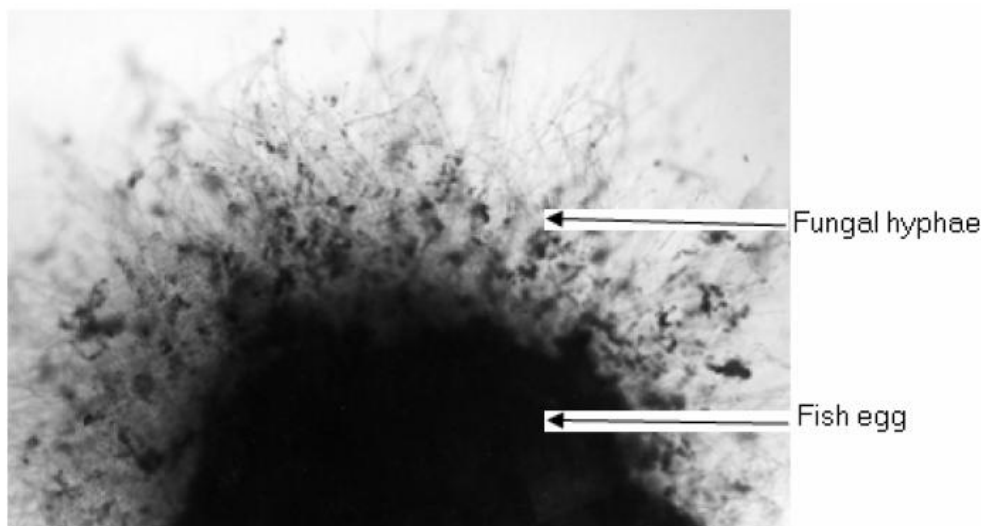


Figure 2. An egg of *Aphanius d. dispar* infected with fungi in the breeding tanks.

Table -2. t-Test: Paired Two Sample for Means of the average eggs of Dispar dispar collected from the two study systems (with cage/ without cage) , ($t < 0.001$)

	With cages	without cages
Mean	2.88	0
Variance	0.179555556	0
Observations	10	10
Hypothesized Mean Difference	0	
df	9	
t Stat	21.49280331	
P(T<=t) one-tail	2.4039E-09	
t Critical one-tail	1.833112923	
P(T<=t) two-tail	4.8078E-09	
t Critical two-tail	2.262157158	

DISCUSSION

The present study shows that breeding of *Aphanius dispar dispar* occurs only in the tanks with cage system and not in the tanks without cage system, probably, because the fish in the tanks without cage system cannibalize on their eggs and fries, if breeding or hatching had occurred in these tanks. In the tanks with cage system, the fish is prevented from reaching to their eggs or fry residing at the bottom of the tank. Both eggs and fries settled down in the tank and don't rise up until they acquire a bigger size, therefore cannibalism by their parents is

avoided. Similar results of cannibalistic behavior of *A. dispar dispar* were reported by Frenkel and Goren (1999) and advised to use cages to prevent cannibalism on fish fry.

The average egg production per tank per week was 2.88 where as hatchability was recorded 41% in the study made with cage system. Performance can be improved in order to produce a large quantity of eggs and a high hatching rate of the eggs. Both figures are low and this may be attributed to the fungal infection of the eggs which causes a high mortality rate among the embryos. The system can be improved through eradication of the fungi in the breeding tanks and the incubation system of the eggs. Fungal infection can be prevented by using ultra violet light to eradicate the fungus and bacteria which may come as a result of fungal infection. The UV system is being used for a long time to disinfect tilapia eggs which enhance productivity of tilapia fry (Rana and Suliman, 1993). The quantity of *A. dispar dispar* can be increased also by stocking more females in the tank in order to produce more eggs. Formalin can also be used to disinfect the eggs in the hatching jars, but it should be used with care in breeding tanks because it may affect the brooder fishes.

Frenkel and Goren (1997) stated that some environmental factors affect reproduction of *A. dispar dispar* and they found that maturation of oocyte increased by increasing the temperature from 18 to 27 °C. The photoperiod also affects the fish spawning. It is reported that the photoperiod (12:12 hrs) is the most suitable for spawning the fish. So in present study the temperature was adjusted to 23-24 °C which is the most suitable temperature for spawning and hatching and the photoperiod was adjusted to 12: 12 hrs day and night which is also suitable for enhanced spawning of the fish. Salinity seems has no effect on spawning of *A. dispar*. Oltra and Todoli (2000) studied the breeding of *A. dispar* at different salinities ranging from 5 to 40 ppt and found no significant difference between spawning at different salinities.

We collect the eggs for incubation on weekly basis and the period needed for hatching the eggs is 10 days. This relatively long period may lead to fungal infection of the eggs in breeding tanks and the hatching jars. If the eggs collection is made more frequently some infection may be avoided.

We conclude that *A. dispar dispar* can be bred in freshwater cage system. Breeding tanks and hatching jars should be disinfected with U V lamps to eradicate the fungal and bacteria infection for mass production of *A. dispar dispar* for restocking of freshwater bodies in Saudi Arabia for biological conservation of the fish and to use them in biological control of mosquito larvae and aquarium uses, which in turn give the fish the economic and recreational importance. *A. dispar dispar* fry should be segregated by size to avoid cannibalism and more fish to be stocked for breeding.

REFERENCES

- Al-Akel, A. S. , Shamsi, M. J. K. and Al-Kahem, H. F. (1987): Selective feeding behavior of the Arabian freshwater fish , *Aphanius dispar*. Pakistani J. Zool., 19 (3): 211-215.
- Al_ kahem, H. F. and Behnke, R. J. (1983): Freshwater fishes of Saudi Arabia. Fauna Saudi Arabia, 5:545-567.
- Al-Kahem-Albalawi, H. F., Al-Ghanim, K. A. , Ahmad, Z., Temraz, T. A. , Al-Akel, A. S. , Al-Misned, F. and Annazri, H. (2008): A threatened fish species (*Aphanius dispar*) in Saudi Arabia: A case study. *Pakistan J. Biol. Sci.*, 11 (19): 2300-2307.

- (EPAA) Environment and Protected Areas Authority, (2003): Fourth International Conservation Workshop for the Threatened Fauna of Arabia. BCEAW/EPAA; Sharjah, UAE. Pp 141.
- Fouda, M. M. (2005): Life history strategies of four small-size fishes in the Suez Canal, Egypt. *J. Fish Biol.*, 46 (4): 687- 702.
- Frenkel, V. and Goren, M. (1997): Some environmental factors affecting the reproduction of *Aphanius dispar* (Ruppell, 1828) , *Hydrobiologia*, 347(13): 197-202.
- Frenkel, V. and Goren, M. (1999): A spawning cage for eliminating predation on larvae of the killifish *Aphanius dispar*. *North American J. Aquacul.*, 61:172–174.
- Froese, R. and Pauly. D. (2009): FishBase.World Wide Web electronic publication. www.fishbase.org, version (04/2009).
- Haas, R. (1982): Notes on the ecology of *Aphanius dispar* (Pisces, Cyprinodontidae) in the Sultanate of Oman. *Freshwater Biol.*, 12 (1): 89 – 95.
- Krupp, F. (1983): Freshwater fishes of Saudi Arabia, *Fauna of Saudi Arabia*, 5: 545-567.
- Krupp, F. (1988): Freshwater fishes of Wadi Batha drainage. *J. Oman Studies, Special Report*, 3: 401-404.
- Lotan, R. (1969): Sodium chloride and water balance in the euryhaline teleost *Aphanius dispar* (Ruppell) (Cyprinodontidae). *Z Vergl Physiol*, 65:455–462.
- Lotan, R. (1971): Osmotic adjustment in the euryhaline teleost *Aphanius dispar* (Cyprinodontidae). *Z Vergl Physiol*, 75: 383–387.
- Lotan, R. and Skadhauge, E. (1972): Intestinal salt and water transport in a euryhaline teleost, *Aphanius dispar* (Cyprinodontidae). *Comp. Biochem. Physiol.*, 42A:303–310.
- Oltra, R. and Todoi, R. (2000): Reproduction of the endangered Killifish *Ahanius iberus* at different salinities. *Env. Biol. Fish*, 57: 113-115.
- Plaut, I. (2000): Resting metabolic rate, critical swimming speed, and routine activity of the euryhaline cyprinodontid, *Aphanius dispar*, acclimated to a wide range of salinities. *Physiol. Biochem. Zool.* 73 (5): 590-596.
- Rana, K. J. and Suliman, E. M. (1993): Artificial incubation of tilapia eggs and fry under semi intensive and intensive aquaculture systems: Proceeding of first international symposium on aquaculture technology and investment opportunities held on 11-14 April, 1993. Organized by KACST and the Ministry of Agriculture. 208-212.
- Skadhauge E. and R. Lotan. (1974): Drinking rate and oxygen consumption in the euryhaline teleost *Aphanius dispar* in waters of high salinity. *J. Exp. Biol.* 60:547 556.

STRUCTURE OF A GLUCAN AND A HETEROPOLYMER FROM THE CELL WALL OF THE YEAST *PHAFFIA* *RHODOZYMA*

**Juliana Negrão Borges^a, Tania Maria Bordin Bonfim^a, Guilherme
Lanzi Sassaki^b, Ricardo Wagner^c, José Domingos Fontana^a, Sandra
Mara Woranovicz-Barreira¹**

^aDepartamento de Farmácia, Universidade Federal do Paraná, R. Prefeito Lothário
Meissner, 632, Curitiba-PR (80210-170), Brazil

^bDepartamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná,
P.O.Box 19046, Curitiba-PR (81531-990), Brazil

^cEscola de Saúde, UNIBRASIL, R. Konrad Adenauer, 442,
Curitiba-PR (82820-540), Brazil

ABSTRACT

Polysaccharides from *Phaffia rhodozyma* SDS-treated cell walls were successively obtained via cold water, hot water, and dimethylsulfoxide extractions. For this, processed yeast cells were grown under a physiological condition of active production of astaxanthin, its characteristic carotenoid metabolite. Afterwards, the extracted fractions were fractionated via freeze-thawing and precipitation with either Fehling reagent or Cetavlon. An α -D-glucan and a heteropolysaccharide were isolated and structurally investigated using monosaccharide composition determination, methylation analysis, and COSY, TOCSY and HMQC techniques of ^{13}C - and ^1H -NMR. The DMSO-extracted and Fehling-purified α -D-glucan was determined to be a linear chain of α -D-(1 $\rightarrow$ 3)-glucopyranosyl units. The hot water-extracted and Cetavlon-precipitated heteropolysaccharide showed a complex structure containing α -D-(1 $\rightarrow$ 4)-mannopyranosyl units-based main chain with side chains containing β -D-(1 $\rightarrow$ 4)-xylopyranosyl units along with D-mannose, D-galactose, and D-glucose units. In addition, these monosaccharides are found to be non-reducing ends of the heteropolysaccharide as well. The cell wall extraction schedule also yielded several minor glucan fractions displaying a positive iodophilic reaction which was abolished after pretreatment with crystalline *Bacillus subtilis* α -amylase. The knowledge of these cell wall features is of importance for the application of whole *P. rhodozyma* cells as single cell protein, improved digestibility, and astaxanthin release.

¹ Corresponding author: sandra@ufpr.br Fax: 55-41-3360-4101, Tel: 55-41-3360-4089.

Keywords: *Phaffia rhodozyma*; yeast cell wall; polysaccharide.

1. INTRODUCTION

The red yeast *Phaffia rhodozyma* was isolated by Herman Phaff in the 1960s [1]. Initially taken as a single genus and species, this yeast taxonomy presents some controversy due to the occurrence of *Phaffia rhodozyma* anamorph(s) and *Xanthophyllomyces dendrorhous* teleomorphs [2]. The yeast has attracted biotechnological interest due to its ability to synthesize as major carotenoid the astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) [3], particularly in its unesterified 3*R*, 3'*R* stereo form, which is one of the natural forms found in many organisms [4]. Astaxanthin is the carotenoid pigment naturally found in many invertebrate and fish, such as salmonids being responsible for the meat characteristic color. These animals cannot synthesize the carotenoids “de novo”, and depend entirely on dietary supplement to achieve the natural pigmentation [5]. Astaxanthin is the preferred pigment for use as colorant in aquaculture, especially as feed supplement in farmed trout, salmon and prawns since it is incorporated more efficiently than any other carotenoids. However, the yeast rigid cell wall severely reduces the intracellular pigment bioavailability. For instance, incorporation of undisrupted yeast in the diets of rainbow trout resulted in low or no pigmentation [3].

The yeast cell wall is responsible for determining the shape and morphology of the cells while affording mechanical protection from the environmental changes [6]. In general, the cell wall is composed of $\beta(1\rightarrow3)$ - and $\beta(1\rightarrow6)$ -D-glucans linked together with highly branched mannoproteins in a complex way [7]. The study of the polysaccharides from cell wall may aid the development of a specific rupture method for the industrial production of free carotenoid. In the present work we have isolated and characterized two polysaccharides from the yeast cell wall and they are described below.

2. EXPERIMENTAL

2.1. Microorganism and Culture Conditions

The anamorphic yeast *Phaffia rhodozyma* (ATCC 24202) was grown in a medium consisting of 20g sucrose/L, 1g yeast extract/L, and 5g peptone/L in 250 mL Erlenmeyer flasks that were agitated in a rotatory shaker at 150 rpm for 48 hours at 24°C for inoculum preparation. This culture was employed to inoculate the batch process of cell production. The batch cultures were grown in a 5 L B. Braum Biotech B bioreactor employing a media with the same composition as in the previous phase. The pH was controlled at $\text{pH } 6.0 \pm 0.2$ by the automatic addition of M NaOH and M H₂SO₄. The temperature was controlled at $24 \pm 0.5^\circ\text{C}$ as well as the agitation rate. Aeration was manually controlled to maintain a dissolved oxygen concentration above 40%. The culture was carried out over a period of 98 hours in the absence of bright light in order to avoid carotenoid photo bleaching. Subsequently, the yeasts were harvested by centrifugation, washed with distilled water and lyophilized.

2.2. Carotenoid Analyses

The carotenoid content of the produced yeast was measured by HPLC. Samples were frozen in liquid nitrogen, pulverized and extracted with acetone in order to remove the carotenoid. After centrifugation, the supernatant was filtrated in 0.2 μm Teflon (PTFE) membrane. The analyses were performed in a Shimadzu LC-10A liquid chromatograph equipped with C18 Vydac 201TP54 4.6 mm x 250 mm column at 30°C (Shimadzu CTO-10A). Liquid phase was methanol : acetonitrile (9:1, v/v). Carotenoid detection was performed by an UV-VIS Shimadzu SPD-10A detector set at 480 nm. Identification of the main carotenoid peak was accomplished using the elution time obtained from a standard solution of astaxanthin and its characteristic λ_{max} at 480 nm.

2.3. Extraction and Fractionation of Cell Wall Polysaccharides

Dry cells (10g) were treated with a 2% aqueous SDS (sodium dodecyl sulphate) at room temperature for 12h (X4) for proteins, nucleic acids, and lipids removal in the supernatant. The resulting precipitate (5g) was extracted with water at room temperature for 24h, followed by centrifugation to give a supernatant, which was precipitated with a mixture of ethanol:water (3:1, v/v), as illustrated in Figure 1. The precipitate was dissolved in hot water, dialyzed and freeze-dried (CWI). This process was repeated 2 times more to yield fractions CWII and CWIII. The remaining residue from the initial cold water extraction was successively extracted 3 times with hot water at 80°C for 24h to give fractions HWI, HWII and HWIII. Finally, fractions DMI, DMII and DMIII were obtained by extracting the remaining residue with DMSO at room temperature for 2h also in 3 sequential cycles. All these fractions were tested for sugar content according to Dubois [8] and afterwards were frozen and thawed to give precipitate (FTP) and supernatant (FTS) subfractions. Samples which showed a higher yield (HW FTS and DM FTP) were treated with Fehling's solution (~40mL) [9]. The resulting complexes (FP) and supernatants (FS) were each neutralized with AcOH, dialyzed against distilled water. This treatment gave rise to the main fraction, originated from mixed DM I, II and III which did not precipitate with Fehling solution (DM FS). The fraction obtained successively from HW I and FS was dissolved in water (~50mL) and mixed with a 3% solution of Cetavlon in water at pH 7.0 (50mL). The supernatant of the Cetavlon precipitate was treated with an aq. solution of $\text{Na}_2\text{B}_4\text{O}_7$ at pH 8.5, which was then adjusted to pH 10.0 and then to 12.0, with aq. NaOH. The resulting precipitates were isolated by centrifugation and decomplexed with 4M aq. NaCl followed by precipitation in ethanol. For the cases where precipitation was observed at pH 8.5, decomplexation was achieved with a 2M aq. AcOH followed by precipitation in ethanol. Here, we have studied the portions that precipitated at pH 10.0 (HW I C10.0).

2.4. Analysis of Monosaccharides Present in Polysaccharides

The polysaccharides were hydrolyzed with 1 M TFA at 100°C for 8h, followed by evaporation to dryness and successive reduction with NaBH_4 and acetylation with

Ac₂O:pyridine (1:1, v/v) [10, 11]. The resulting alditol acetates were extracted with CHCl₃ and analyzed by GC-MS, using a DB-225 or DB-23 capillary column (30mX0.25mm i.d.) using an initial temperature of 50°C, a heating rate of 40°C/min, a final temperature of 220°C, and a FID detector.

The uronic acid content was determined by an improved m-hydroxy-biphenyl colorimetric method [12].

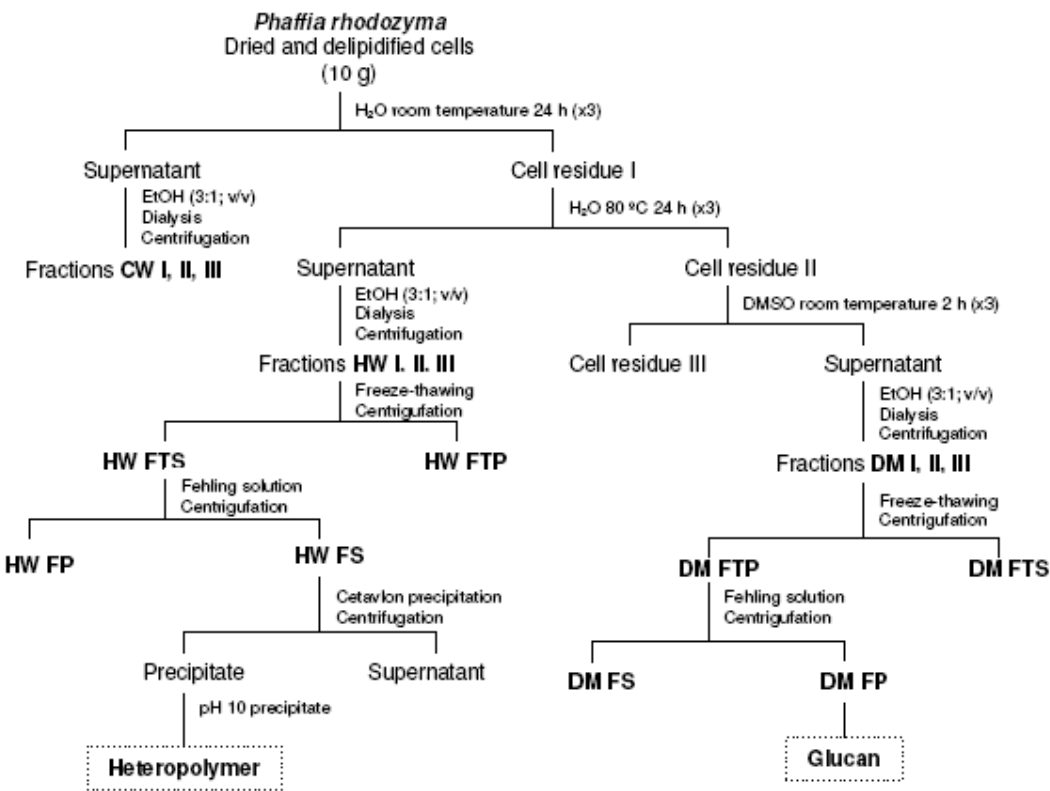


Figure 1. Extraction, fractionation and isolation of polysaccharides from *Phaffia rhodozyma*.

2.5. Methylation Analysis of Polysaccharides

Five milligrams of each purified fraction were per-*O*-methylated using powdered NaOH in DMSO-iodomethane [13]. The per-*O*-methylated derivatives were treated with 0.5mL of 50% (v/v) or 72% (w/v) sulfuric acid (1h, 0°C) and the resulting solution diluted with H₂O (4mL) and completely hydrolyzed at 100°C for 18h. The solution was neutralized with BaCO₃ and filtered. The filtrate was evaporated to dryness and subsequently treated with NaBD₄ in order to convert the partially *O*-methylated aldoses to alditol derivatives, which were analyzed by GC-MS as described in Section 2.4.

2.6. Partial Acid Hydrolysis of Heteropolymer

Twenty milligrams of fraction HW I C10.0 was partially hydrolyzed with 0.1M aq. trifluoroacetic acid, at 100°C for 2h. The acid was removed by freeze-drying. The resulting material was redissolved in distilled water, and then retained on dialysis with a Mw 8kDa cut-off membrane (13 mg).

2.7. HPSEC Analysis

The elution profile of water-soluble fraction was determined by high performance size-exclusion chromatography (HPSEC), using a Waters 510 HPLC pump at 0.6mL/min with four gel permeation columns in series with exclusion sizes of 1×10^6 , 4×10^5 , 8×10^4 , and 5×10^3 Da, using a refraction index detector. The eluent employed was 0.1M/L aq. NaNO_2 containing 200 ppm aq. NaN_3 . Prefiltered samples (0.20 μm , Millipore) were injected at the concentration of 1mg/mL.

2.8. Nuclear Magnetic Resonance Spectroscopy

NMR spectra were obtained with a DRX Avance 400MHz Bruker spectrometer. ^{13}C (100.6 MHz) and ^1H NMR (400.13) spectra were acquired at 70 or 50°C, with the samples being dissolved in D_2O or in DMSO-d_6 depending on their solubility. Chemical shifts are expressed in δ ppm relative to acetone at δ 30.20 and 2.224 for ^{13}C and ^1H signals, respectively.

2.9. Enzymatic Degradation of Iodophilic α -Glucan Minor Fractions

Minor polysaccharide fractions (5 mg/mL in water) were assayed before and after pretreatment with crystalline α -amylase from *Bacillus subtilis* (Sigma; 2 mg/mL) at 35°C overnight with 1:50 diluted I_2 / KI (1:10, g%) regarding the bluish color given by amylose-like polysaccharides. The duplicated non stained enzymatic reactions were stopped by the addition of 3 volumes of ethanol and the evaporated supernatants chromatographed in silica gel chromatoplates with isopropanol:ethyl acetate:water (7:1:2, v/v) (1.5 x ascending developments) as mobile phase for the detection of glucose and maltosaccharides after revealing with hot orcinol : sulfuric acid : methanol (1 : 9 : 90).

3. RESULTS AND DISCUSSION

The carotenoid content of the cultivated cells from *Phaffia rhodozyma* was 163.33 μg astaxanthin/g of dried yeast cell, demonstrating a satisfactory pigment production in the grow medium used [14]. Although carotenoid production is not the aim of this work, this result confirmed the healthy growth of the microorganism, ensuring that the cell wall also

corresponded to a full and functional cell envelope. The lyophilized cells had their lipids, proteins and nucleic acids removed with 2% aq. SDS (unpublished results). The remaining material containing cell walls was then subjected to cold water, hot water and DMSO extractions, followed by purification of the component polysaccharides (Figure 1).

3.1. Isolation and Characterization of Glucan Component

The fractions extracted with DMSO showed almost identical contents of xylose, mannose, galactose and glucose (acid monosaccharides were not found), with decreasing and respective yields of 6.5, 2.8 and 0.7% for fractions I, II and III as referred to the starting material. Glucose was the predominant component (~82 mol%). Therefore, all DMSO fractions were combined and subjected to freeze-thawing cycles. The water insoluble fraction (FTP) was obtained in a higher yield (8.9%), with decreased contents of xylose and galactose in relation to crude fraction (DM).

In order to obtain a purified glucan, FTP was submitted to Fehling fractionation, which yielded 2 portions: soluble (DM FS; 4.5% yield) and precipitated material (DM FP; 3.3% yield). Both fractions showed high contents of glucose, 84.9 and 75.6 mol%, and DM FS consisted of a structure similar to pseudonigeran α -D-glucan, with a typical ^{13}C NMR spectrum (Figure 2) [15, 16]. It contained six major signals of the glucan at δ 99.6 (C-1), 82.8 (C-3), 71.9 (C-5), 70.8 (C-2), 69.5 (C-4) and 60.3 (C-6). Methylation analysis showed 2,4,6-tri-*O*-methyl-glucose as main *O*-partially methylated derivative (80mol%).

Glucans have been found in various species of yeasts. It is the first time that this particular structure is described in *Phaffia rhodozyma*, although other authors have already suggested the presence of an α -glucan in its cell wall [2]. The same glucan was firstly described in *Aspergillus niger* then isolated with hot water, and also in other fungus species (*Polyporus betulinus*, *Tremella mesenterica*, *Aspergillus nidulans*, *Schizophyllum communis* and in *Cryptococcus* and *Schizosaccharomyces* genus) [17]. In *Schizosaccharomyces pombe*, the α -(1 $\rightarrow$ 3)-glucan was observed in the surface of the cell wall and in regions adjacent to the cell membrane [18].

3.2. Isolation and Characterization of Heteropolymer Component

The mother liquors of the freeze-thawing process following hot water extraction (HW FTS I, II and III) were further fractionated. They were treated with Fehling solution to give traces of precipitates and the main fraction was found in the supernatant (HW FS I, II, III). Their monosaccharide compositions were significantly different, where mannose was the principal monosaccharide in the fraction I (57 mol%), and glucose in the fractions II and III (44 and 69 mol% respectively).

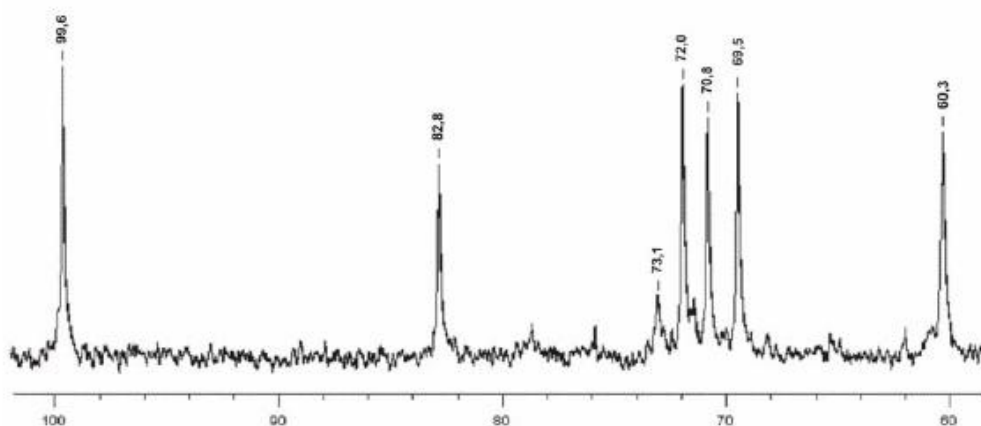


Figure 2. ^{13}C NMR spectrum of $\alpha(1\rightarrow3)\text{-D-glucan}$ (DM FS fraction) in $\text{Me}_2\text{SO-d}_6$ at 70°C : chemical shifts in δ ppm.

Fraction HW FS I was further fractionated with Cetavlon; no precipitation occurring at pH 7.0, agreeing with no acidic monosaccharide being detected. Precipitation occurred mainly at pH 10.0 in the presence of borate.

HPSEC-MALLS analysis of HW I C10.0 gave a homogeneous profile, with $M_w 87.3 \times 10^3$ g/mol and $dn/dc=0.143$. It contained xylose, mannose, galactose, and glucose in a 22:67:7:4 molar ratio, suggesting a xylomannan main structure.

Methylation analysis of HW I C10.0 (Table 1) showed a highly branched structure based on derived partially *O*-methylated alditol acetates (GC-MS) with a high proportion of nonreducing end-units of Xyl (15.1%), Man (10.9%) and Gal (10.9%), 4-*O*-substituted (12.0%) Xylp and also 4-*O*-substituted (17.4%) Manp.

The ^{13}C NMR spectrum of the heteropolymer (Figure 3) corresponded to a highly complex molecule with many C-1 signals, which indicated predominant branched structures with nonreducing end-units of $\alpha\text{-D-Glcp}$ (δ 99.6), $\alpha\text{-D-Manp}$ (δ 101.7), $\beta\text{-D-Xylp}$ (δ 102.8), and $\beta\text{-D-Galp}$ (δ 103.4)[15, 19].

In order to elucidate the core of the heteropolymer, a partial acid hydrolysis was carried out and removed the majority of the Xylp units. The product, PH HW I C10, retained on dialysis, contained Xyl (10%), Man (80%), Gal (6%) and Glc (4%). Methylation analysis (Table 1) indicated that the Xylp units were mainly present as nonreducing end units, along with Galp. The increasing content of 2,3,4,6-Me₄Man after hydrolysis indicated that the non-reducing end units of Xylp were linked to Manp, which were exposed now as nonreducing end units. Also it was found increased contents of Man 2-*O*-, Man 4- and Gal 6-*O*-substituted. These units probably are from the internal core of the original polymer, the increased values resulting from the reduced size of the molecule after partial hydrolysis. ^{13}C NMR examination (Figure 4) agreed with the removal of Xylp units, as their intensity decreased in relation to $\alpha\text{-Manp}$ units, when compared with the original polymer (Figure 3). The anomeric carbon α -linkage confirmation was obtained by the analysis of the coupled HMQC assay, where it was found a 178 Hz coupling relative to C1 and H1 of the Manp units.

Table 1. Partially O-methylated alditol acetates formed on methylation and GC-MS procedure of heteropolysaccharide (HW I C 10.0 fraction) and its partial hydrolysis product (HP HW I C 10.0 fraction)

O-Me-alditol acetate ^a	Fractions (molar % ^b)		Linkage type ^c
	original	partial hydrolysis	
2,3,4-Me ₃ -Xyl	15.1	6.6	Xylp-(1→
2,3,4,6-Me ₄ -Man	10.9	16.9	Manp-(1→
2,3,4,6-Me ₄ -Glc	5.3	-	Glc p-(1→
2,3,4,6-Me ₄ -Gal	10.9	6.4	Galp-(1→
2,3-Me ₂ -Xyl	12.0	5.5	→4)Xylp-(1→
3,4,6-Me ₃ -Man	7.3	8.9	→2)Manp-(1→
2,4,6-Me ₃ -Man	2.3	-	→3)Manp-(1→
2,3,6-Me ₃ -Man	17.4	31.2	→4)Manp-(1→
2,3,4-Me ₃ -Gal	2.4	5.6	→6)Galp-(1→
2,3,6-Me ₃ -Glc	-	2.7	→4)Glc p-(1→
2,6-Me ₂ -Man	2.1	3.5	→3,4)Manp-(1→
3,6-Me ₂ -Man	5.2	6.0	→2,4)Manp-(1→
2,3-Me ₂ -Glc	1.9	-	→3,4)Glc p-(1→
3,4-Me ₂ -Man	4.6	3.7	→2,6)Manp-(1→
4-Me ₁ -Glc	2.0	3.1	→2,3,6)Glc p-(1→

^a O-Me-alditol acetates obtained by methylation analysis, followed by hydrolysis, reduction with NaBD₄ and acetylation, analyzed by GC-MS (column DB-225).

^b % of peak area relative to total peak area.

^c Based on derivated O-methylalditol acetates.

The C-1 region can be interpreted in terms of a principal structure with a (1→4)-linked α -D-Manp main chain (C-1: δ 101.7; C-2: δ 69.4; C-3: δ 73.5; C-4: δ 78.5; C-5: δ 75.9; C-6: δ 61.0) partially substituted at O-2 and/or O-6 with Manp (δ 101.7), β -D-Galp (δ 103.4), α -D-Glc p (δ 99.3) and internal units of (1→4)-linked Xylp (δ 102.7). These units were assigned in the ¹³C NMR spectrum: δ 72.8 (C-2), δ 75.1 (C-3), δ 77.0 (C-4 linked) and δ 65.2 (C-5) [15, 20, 21].

The supposed linkage of main chain are in agreement with the increased value of 2,3,6-Me₃-Man derivative after hydrolysis (17.4% to 31.2%). Polysaccharides structures containing α -(1→2) or α -(1→6) Manp units main chains have been described in several Basidiomycete yeast such as *Torulopsis apicola*, *T. lactis-condensi* and *Trichosporon aculeatum* [22].

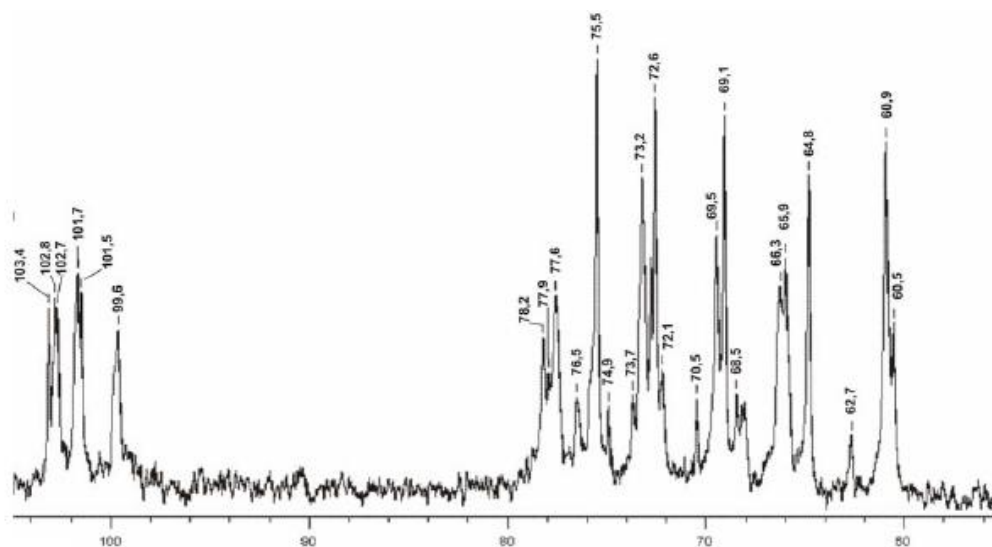


Figure 3. ^{13}C NMR spectrum of the heteropolymer (HW I C 10.0 fraction) in D_2O at 50°C : chemical shifts in δ ppm.

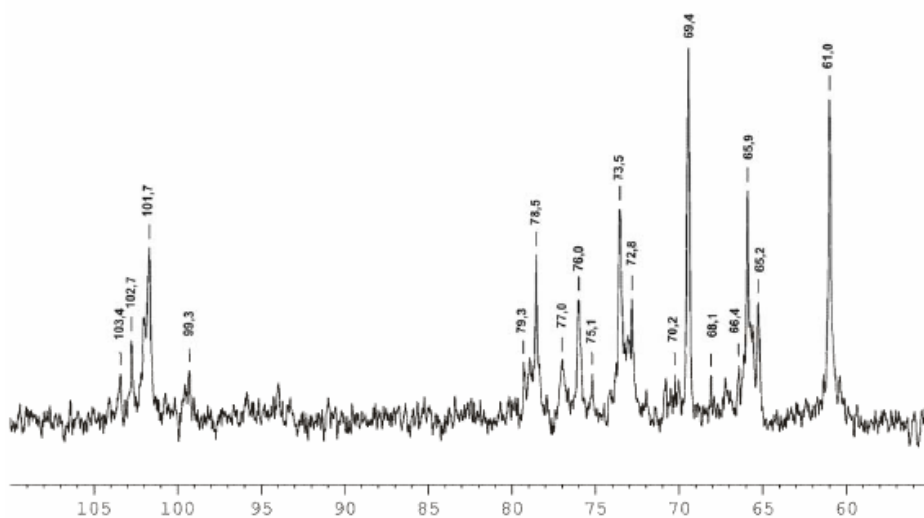


Figure 4. ^{13}C NMR spectrum of the heteropolymer after partial acid degradation (PH HW I C 10.0 fraction) in D_2O at 50°C : chemical shifts in δ ppm.

Several minor fractions from the yeast cell wall such as HW FTP, DM FS, DM FP, and HW FS which did not precipitate with Cetavlon, displayed a bluish reaction when assayed with iodine/potassium iodide. This color pattern was completely abolished after pre-incubation with crystalline α -amylase from *Bacillus subtilis*. In order to confirm the α -1,4-glucan nature of these fractions the corresponding ethanolic supernatants were chromatographed aside the pertinent standards. In order of decreasing amounts the fragments of the amylolytic enzyme action were maltotriose, maltopentaose, maltose, maltotetraose, and glucose. Hence, the yeast

cell wall despite de extensive washing of cytoplasmatic components with SDS still retains an α -amylose-like component. The alternative explanation is the presence of an iodophylic glucan as a true minor component of the native cell wall. Grün et al [23] have shown that alpha-glucan in fission yeast *Schizosaccharomyces pombe* also contains stretches of α -1,4-glucan.

4. CONCLUSION

Since astaxanthin accumulation in *Phaffia rhodozyma* is solely an intracellular event and since there is no convincing methodology for cheap rupture of the cell wall when dealing with formulations designated to human kind and animal consumption, knowledge gain on the yeast cell wall is biotechnologically important in order to ensure a prompt release of the antioxidant astaxanthin and other intracellular and less soluble components. The present molecular characterization of homo- and heteropolysaccharides fulfill such a purpose.

ACKNOWLEDGMENTS

The authors would like to thank the Brazilian agencies CAPES and PRONEX-CARBOIDRATOS for financial assistance and Dr. Marcelo Maraschin for carotenoid assay.

REFERENCES

- [1] Miller, M.W.Y.; Oneyama, M.; Soneda, M. (1976) *Phaffia*, a new yeast genus in the *Deuteromycotina* (*Blastomycetes*), *Int. J. Syst. Bacteriol.* 26, 286-291.
- [2] Fell, J.W. and Blatt, G.M. (1999) Separation of strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis, *J. Ind. Microbiol. Biotechnol.* 23, 677-681.
- [3] Johnson, E.A. and An, G-H. (1991) Astaxanthin from microbial sources, *Crit. Rev. Biotechnol.* 11, 297-326.
- [4] Andrewes, A.G.; Phaff, H.J.; Starr, M.P. (1976) Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast, *Phytochem.* 15, 1003-1007.
- [5] Storebakken, T. and No, H.K. (1992) Pigmentation of rainbow-trout, *Aquacult.* 100, 209-229.
- [6] Gacto, M.; Vicente-Soler, J.; Cansado, J.; Villa, T.G. (2000) Characterization of an extracellular enzyme system produced by *Micromonospora chalybeata* with lytic activity on yeast cells, *J. Appl. Microbiol.* 88, 961-967.
- [7] Lipke, P.N. and Ovalle, R. (1998) Cell wall architecture in yeast: New structure and new challenges, *J. Bacteriol.* 180, 3735-3740.
- [8] Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. (1956) Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28, 350-356.
- [9] Jones, J.K.N. and Stoodley, R.J. (1965) Fractionation using copper complexes, *Met. Carbohydr. Chem.* 5, 36-38.

- [10] Wolfrom, M.L. and Thompson, A. (1963a) Reduction with sodium borohydride, *Met. Carbohydr. Chem.* 2, 211-215.
- [11] Wolfrom, M.L. and Thompson, A. (1963b) Acetylation, *Met. Carbohydr. Chem.* 2, 211-215.
- [12] Filisetti-Cozzi, T.M.C.C. and Carpita, N.C. (1991) Measurement of uronic- acids without interference from neutral sugars, *Anal. Biochem.* 197, 157-162.
- [13] Ciucanu, I. and Kerek, F.A. (1984) A simple and rapid method for the permethylation of carbohydrates, *Carbohydr. Res.* 13, 209-217.
- [14] Vázquez, M.; Santos, V.; Parajó, J.C. (1997) Effect of the carbon source on the carotenoid profiles of *Phaffia rhodozyma* strains, *J. Ind. Microbiol. Biotechnol.* 19, 263-268.
- [15] Gorin, P.A.J. (1981) Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides, *Adv. Carbohydr. Chem. Biochem.* 38, 13-104.
- [16] Sugawara, T.; Takahashi, S. ; Osumi, M.; Ohno, M. (2004) Refinement of the structures of cell-wall glucans of *Schizosaccharomyces pombe* by chemical modifications and NMR spectroscopy, *Carbohydr. Res.* 339, 2255-2265.
- [17] Gorin, P.A.J. and Barreto-Bergter, E. The chemistry of polysaccharides of fungi and lichens, in: G. O. Aspinall, (Ed), *The Polysaccharides*, New York: Academic Press, Inc., 1983, pp. 365-409.
- [18] Pérez, P. and Ribas, J.C. (2004) Cell wall analysis, *Meth.*, 33, 245-251.
- [19] Gorin, P.A.J. and Mazurek, M. (1975) Further studies on the assignment of signals in ¹³C magnetic resonance spectra of aldoses and derived methylglycosides, *Can. J. Chem.* 53, 1212-1222.
- [20] Kovac, P. ; Hirsch, J.; Shashkov, A.S.; Usov, A.I.; Yarotsky, S.V. (1980) C-13-NMR spectra of xylo-oligosaccharides and their application to the elucidation of xylan structures, *Carbohydr. Res.* 85, 177-185.
- [21] Habibi, Y. and Vignon, M.R. (2005) Isolation and characterization of xylans from seed pericarp of *Argania spinosa* fruit, *Carbohydr. Res.* 340, 1431-1436.
- [22] Gorin, P.A.J. and Spencer, J.F.T. (1966) Exocellular alginic acid from *Azotobacter vinelandii*, *Can. J. Chem.* 44, 993-998.
- [23] Grün, C. H.; Hochstenbach, F.; Humbel, B. M.; Verkleij, A. J.; Sietsma, J. H.; Klis, F. M.; Kamerling, J. P.; Vliegthart, J. F. (2005) The structure of cell wall alpha-glucan from fission yeast, *Glycobiol.* 15, 245-257.

PRIMARY IMMUNE RESPONSE AND PARASITE DISSEMINATION IN CANINE VISCERAL LEISHMANIASIS

***P.S.T.Veras¹, W.L.C.dos-Santos¹, A, T.F.lmeida¹,
and C.A.T. Rodrigues²***

¹Laboratório de Patologia e Biointervenção, CPqGM, FIOCRUZ BA/Brazil,

²Departamento de Morfofisiologia da UFMS-MS/Brazil

ABSTRACT

Zoonotic visceral leishmaniasis is a re-emerging disease caused by *L. infantum/L. chagasi*. The disease is transmitted by phlebotominae sand flies and dogs are the main urban reservoir of the parasite. In the natural history of *L. chagasi* infection in dog, named canine visceral leishmaniasis (CVL), following transmission, the parasites multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog. In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites. After infection, some dogs develop an impaired cell-mediated immune response that permits parasite dissemination and tissue lesion formation (symptomatic dogs), whereas others control parasite proliferation and dissemination to the different tissues (asymptomatic dogs). These infected dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection. However, as the disease progresses in susceptible dogs, these responses diminish. The cellular basis and mechanisms for the development of T-cell unresponsiveness in CVL are not understood fully. In the present review it will be discussed the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL.

Keywords: *Leishmania chagasi*, canine visceral leishmaniasis, macrophage, spleen, parasite dissemination.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a chronic and frequently lethal disease caused by *L. (L.) donovani* and *L. (L.) infantum* in the Old World and *L. chagasi* in the Americas (Desjeux, 2001a, b, 2004; Palatnik-de-Sousa *et al.*, 2001; WHO, 1999). Recent evidence shows that the *L. (L.) chagasi* strains could not be distinguished from *L. (L.) infantum*, indicating a recent geographical separation, and that *L. (L.) infantum* and *L. (L.) chagasi* are two names for the same species (Mauricio *et al.*, 2000). VL is mostly a rural disease, with a domestic or peridomestic occurrence. In Brazil, however, urbanization of visceral disease has been found in towns or in the outskirts of large cities such as Belo Horizonte, Montes Claros, Rio de Janeiro, Salvador, and Fortaleza (Palatnik-de-Sousa *et al.*, 2001).

The disease is lethal if not treated early after the onset of the symptoms. Dogs present several signs, and progressive suppression of the cellular immune response. These parasites live inside monocytes and macrophages of lymphoid organs such as the spleen, lymph nodes, bone marrow, and liver. Their biological cycle alternates between the amastigote form in vertebrate host and the promastigote form in the gut of the insect sand fly vector (Baneth and Aroch, 2008). The present review aims to discuss the local immune response in skin, other affected organs, and cellular compartments as well as the possible mechanisms involved in dissemination of the *L. chagasi* infection in the dog model of VL. These mechanisms probably account to development of T-cell unresponsiveness, and disease progression in susceptible dogs.

EPIDEMIOLOGY

The American and European forms of zoonotic VL show several similarities, as both are canid zoonoses that affect mainly children and young human adults (Palatnik-de-Sousa *et al.*, 2001); however, higher seroprevalences have been reported in South America (Ashford *et al.*, 1998; Zerpa *et al.*, 2000). It is well accepted that canids are the most common reservoirs of viscerotropic species causing zoonotic VL in the Mediterranean region, Asia, North Africa, and South America (Moreno and Alvar, 2002), playing a central role in the transmission cycle to humans by phlebotominae sand flies. CVL is a major veterinary and public health problem, and it has also been suggested as a good model for investigating the pathogenesis of human VL (Quinnell *et al.*, 2001b).

CVL has a high prevalence of infection, involving as much as 63%–80% of the dog population, and it is accompanied by a lower rate of apparent clinical disease (Berrahal *et al.*, 1996; Leontides *et al.*, 2002; Solano-Gallego *et al.*, 2004). Previous epidemiologic studies have indicated that about half of the dogs possessing anti-leishmanial antibodies exhibit no clinical signs of disease (Abranches *et al.*, 1991; Acedo-Sanchez *et al.*, 1998; Fisa *et al.*, 1999; Mauricio *et al.*, 1995). Interestingly, in a recent study in Northeast Brazil, a higher prevalence of positive dogs presenting no clinical signs of the disease was detected in the metropolitan when compared to a rural area (Alvar *et al.*, 2004; Berrahal *et al.*, 1996; Queiroz *et al.*, 2008; Solano-Gallego *et al.*, 2001). Cumulative evidence shows that: (1) infection in the canine population in endemic areas is widespread, and the rate of infected dogs is much higher than the fraction that shows clinical illness; (2) infection spreads quickly and extensively among the

dog population when environmental conditions for transmission are optimal (Baneth *et al.*, 2008); and (3) removal or elimination of infected dogs from endemic areas would be followed by immediate substitution of susceptible dogs (Dye, 1996).

Dye (1996) and Burattini and others (1995) have shown that the dog population in endemic areas is composed of four mutually exclusive groups: those susceptible, those resistant, those susceptible that become latent after sand fly bite (asymptomatic), and those infectious to sand flies that emerge from latent dogs at a constant rate (Dye, 1996). Dogs born resistant do not become infectious to sand flies or develop the disease, but do become seropositive after sand fly bite. Such animals include seropositive, non-infectious, asymptomatic dogs from endemic areas that are able to maintain an effective cellular immune response against the parasite. Alvar and others demonstrated that naturally infected asymptomatic seropositive dogs (resistant or latent) are infectious to sand flies (Alvar *et al.*, 1994). Dye also refers to a previous cohort study that used xenodiagnosis to show that infected dogs became infectious to sand flies after a median period of approximately 200 days; although several dogs died of clinical VL, the data indicate that infectiousness was unrelated to the severity of symptoms (Dye, 1996). This model demonstrated that targeting control measures at infectious dogs has a potential impact to reducing transmission. Conversely, infectiousness to sand flies has been shown to be positively associated with antibody titers detected by an enzyme-linked immunosorbent assay (ELISA) and to the intensity of skin disease (dermatitis, alopecia, and chancres) (Courtenay *et al.*, 2002).

In the natural history of *L. chagasi* infection in dog, following transmission the parasites initially multiply in macrophages in the skin at the site of infection. From this localized cutaneous infection, the parasite can disseminate via lymphatic or blood vessels, infecting macrophages of other organs such as the bone marrow, lymph node, liver and spleen, as well as the kidneys and gastrointestinal tract of the dog (Reis *et al.*, 2006). In these naturally infected dogs, the outcome of CVL can vary considerably and probably correlates with the capacity of local skin cells to control parasite infection. CVL clinical manifestations are associated with distinct patterns of immune responses to *Leishmania* parasites (Cardoso *et al.*, 1998; De Luna *et al.*, 2000; Martinez-Moreno *et al.*, 1995; Pinelli *et al.*, 1994; Pinelli *et al.*, 1999; Santos-Gomes *et al.*, 2002; Solano-Gallego *et al.*, 2000). After infection and before seroconversion, dogs infected with *L. chagasi* present with enlarged lymph nodes and dermatitis, without signs of visceral leishmaniasis or changes in behavior. This phase is followed by dissemination of the infection and clinical findings, including loss of appetite, fever, weight loss, alopecia, skin ulceration, onychogryphosis, keratoconjunctivitis, uveitis, bleeding, diarrhea, neuralgia, polyarthritis, interdigital ulceration, and kidney insufficiency (Abranches *et al.*, 1991; Bettini *et al.*, 1986; Molina *et al.*, 1994).

INITIAL IMMUNE RESPONSE

L. chagasi infection initiates when the parasite is inoculated into the skin by a female phlebotominae that probes the skin for blood (Rogers *et al.*, 2004). Incoming and resident phagocytes exit the blood vessels and become infected with the parasites (Moll *et al.*, 1993; Santos-Gomes *et al.*, 2000; Wilson *et al.*, 1987). Based on *in vitro* and *in vivo* animal models of visceral leishmaniasis, it is widely accepted that macrophages play a central role in the

control of *Leishmania* infection. Most of these studies have involved human or murine monocytes/macrophages (Bodman-Smith *et al.*, 2002; Gomes *et al.*, 2000; Murray, 2001), and only a few *in vitro* studies used canine macrophages and *L. chagasi* (Bueno *et al.*, 2005; Gonçalves *et al.*, 2005; Sampaio *et al.*, 2007). Previously, Gonçalves and collaborators (2005) demonstrated that the frequency of peritoneal macrophages from naturally infected dogs expressing the monocyte surface molecules CD11b or CD18 significantly drops upon interaction with *L. chagasi*. More recently, Sampaio and collaborators (2007) showed that monocytes from naturally-infected animals compared to those from experimentally-infected ones are significantly more capable of binding to *Leishmania* promastigotes. Using peripheral monocytes from these naturally *L. chagasi*-infected dogs, the authors demonstrated that these cells display a higher frequency of CD11b-positive monocytes when obtained from peripheral blood. Similar to the previous study performed by Gonçalves and collaborators (2005) the frequency of macrophages expressing CD11b or CD18 has been shown to drop significantly upon interaction with *Leishmania*, and this decrease is more accentuated when *Leishmania* is incubated with exogenous serum (Sampaio *et al.*, 2007). The authors propose that downregulation of these receptors may be related to two mechanisms, they can be occupied by *Leishmania*, or the receptor complexes can be internalized after *Leishmania*-macrophage interaction (Sampaio *et al.*, 2007).

After phagocytosis, *Leishmania* promastigotes transform into amastigotes, which can survive inside macrophages. Some genetic alterations have been related to this event. The *Slc11a1* (NRAMP) protein acts as a proton/divalent cation antiporter, which controls the replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagosome (Gruenheid *et al.*, 1997). The *Slc11a1* gene also regulates macrophage function – including upregulation of chemokine and cytokine genes such as TNF and interleukin-1 β and increased expression of inducible nitric oxide synthase (iNOS) (Blackwell *et al.*, 2001). Polymorphisms in the *Slc11a1* gene have been associated with CVL in dogs of different breeds (Sanchez-Robert *et al.*, 2005). In 164 dogs, 24 polymorphisms were found in the *Slc11a1* gene and 3 polymorphisms were associated with an increased risk for CVL (Sanchez-Robert *et al.*, 2008). Among these, two were single nucleotide polymorphisms (SNP) in the *Slc11a1* promoter region that disrupted putative transcription factor binding sites. These types of SNPs in the canine *Slc11a1* gene promoter suggest a possible role of differential *Slc11a1* gene expression that can interfere with *Slc11a1* function and/or its interaction with many other genes, contributing to CVL susceptibility (Sanchez-Robert *et al.*, 2005; Sanchez-Robert *et al.*, 2008).

COMPARTMENTAL IMMUNE RESPONSE

Skin

From the original site of infection in the skin, amastigotes disseminate throughout the body, causing lesions in different tissues such as the lymph node, liver, spleen, gut, bone marrow and, in dogs, mainly other sites of the skin (Barrouin-Melo *et al.*, 2004; dos-Santos *et al.*, 2004; Reis *et al.*, 2006). In the last few years, a growing number of systematic works provided important contributions to our understanding of the histopathological alterations that

occur in these target organs (Brachelente *et al.*, 2005; Giunchetti *et al.*, 2007; Giunchetti *et al.*, 2008a; Giunchetti *et al.*, 2008b; Lage *et al.*, 2007; Santana *et al.*, 2008; Solano-Gallego *et al.*, 2007; Strauss-Ayali *et al.*, 2007).

Some studies evaluated histological alterations that occur in dog skin in response to *Leishmania* infection (dos-Santos *et al.*, 2004; Brachelente *et al.*, 2005; Solano-Gallego *et al.*, 2007). In a previous study, the histological pattern and parasite load were investigated in clinically normal skin of *Leishmania*-infected dogs (Solano-Gallego *et al.*, 2004). Two groups of *Leishmania*-infected dogs, symptomless animals that, although seronegative or only mildly seropositive, provided positive PCR results for *Leishmania* in the skin and a group of clinically affected dogs that were highly seropositive and PCR-positive were compared. The muzzle skin of symptomless dogs had no demonstrable microscopic lesions or amastigotes. This, together with the positive PCR results for *Leishmania*, indicates that the number of parasites in skin samples from the muzzle must have been very low. The most severe lesions and the greatest parasite loads were located around hair follicles, mainly around the isthmus, associated with the middle vascular plexus of the dermis. This finding suggested hematogenous dissemination of the parasite and tropism for the skin (Solano-Gallego *et al.*, 2007). In conclusion, the results of this study cast doubt on the relevance of infected but symptomless dogs in the epidemiology of canine leishmaniasis (Solano-Gallego *et al.*, 2007).

In another recent study (Brachelente *et al.*, 2005), the question of whether a correlation exists between the number of parasites, the histological response, and the expression of cytokines produced by CD4⁺ Th (Thelper)-2 and Th-1 lymphocytes in lesional skin of naturally infected dogs was assessed. To achieve this objective, the authors evaluated the mRNA expression of canine cytokines such as IL-4, IL-13, TNF- α , and IFN- γ by real-time RT-PCR (qRT-PCR) to determine the cellular immune response in lesional skin biopsies from naturally infected dogs. qPCR was used to determine the number of *Leishmania* in paraffin-embedded tissue sections, and this was compared with cytokine expression in tissue lesions. In *Leishmania*-infected dogs, IL-4, TNF- α , and IFN- γ mRNA production were significantly higher than in controls. Furthermore, dogs with a high *Leishmania* burden demonstrated significantly higher IL-4 expression, whereas no difference was noted with regard to expression of other cytokines. By comparing the pattern of inflammation and cytokine expression, a clear trend became evident, that levels of IL-4, TNF- α , and IFN- γ were elevated both in biopsies with a periadnexal nodular pattern and in biopsies where the severity of the periadnexal infiltrate was equivalent to that of the perivascular interstitial infiltrate. Expression of IL-4, IL-13, and TNF- α was slightly increased in biopsies in which plasma cells prevailed over lymphocytes, whereas expression of IFN- γ was moderately higher when lymphocytes were predominant. In summary, this study demonstrates that the local immune response in naturally occurring leishmaniasis includes Th-1 as well as Th-2 cytokine subsets. Furthermore, the increased expression of the Th2-type cytokine IL-4 associated with both severe clinical signs and a high parasite burden in skin lesions connects severity of the disease to a Th-2-type of immune response (Brachelente *et al.*, 2005).

From Skin to other Organs

A variety of adhesion molecules are involved in phagocyte adherence to the extracellular matrix and cells of the connective tissue (Carlos and Harlan, 1994). Using adhesion blocking assays, our group observed that adherence of non-infected mononuclear phagocytes to the inflamed connective tissue is mediated by beta-1 and beta-2 integrins (Carvalho *et al.*, 2004). Flow cytometry experiments showed no consistent changes in the expression of several integrins on the surface of infected murine phagocytes (Carvalho *et al.*, 2004; Pinheiro *et al.*, 2006). These data suggest that infected and non-infected phagocytes expressed similar amounts of integrins, even though adherence of the former cells to the connective tissue was diminished (Carvalho *et al.*, 2004). CCR1 and CCR7 have been shown to be upregulated about two-fold compared to the control group (Steigerwald and Moll, 2005) and, after infection, there is a decrease in CCR4 and CCR5 expression on phagocytes infected with *L. amazonensis* (Pinheiro *et al.*, 2006). Another factor that may differentially interfere with the migration capability of infected cells is parasite burden. There is an inverse relationship between the degree of infection and the adhesive capability of infected cells. Although infection with small numbers of *Leishmania* does not reduce phagocyte adhesion to connective tissue, connective-tissue adhesion by infected phagocytic cells reaches levels of 20-30% that observed for non-infected cells under conditions of high parasite burden (Pinheiro *et al.*, 2006). These data are in accordance with evidence suggesting that heavily *Leishmania*-infected phagocytes present a wide spectrum of suppressive changes, including changes in B7 expression, impaired integrin function, and inhibition of the expression of a variety of genes in mononuclear phagocytes (Buates and Matlashewski, 2001).

We still have a long way to go to understand the mechanisms that control parasite dissemination in dogs. It appears, however, that animals exhibiting more strict control of parasite burden develop a more limited pattern of differential dissemination of infected cells. As recently shown by Reis and collaborators (Reis *et al.*, 2006), higher parasite burden is found in the skin and spleen than in the bone marrow, liver, and lymph nodes of infected healthy animals (Reis *et al.*, 2006). The parasite distribution tends to be more uniform among tissues of unhealthy animals, and parasitism tends to be more intense in the spleen of animals with more severe disease.

In some visceral leishmaniasis-endemic areas of Brazil, the skin of dogs is prone to be in an almost permanent inflammatory state, not always related to *Leishmania* infection. In a series based on the study of stray dogs from the streets of Jacobina (Bahia state, Brazil), 81% of the dogs without evidence of *Leishmania* infection had inflammatory infiltrates in the ear skin (dos-Santos *et al.*, 2004). Pucheu-Haston and collaborators (Pucheu-Haston *et al.*, 2006) showed that inflammation causes dog skin to function as a source of chemoattractants and favors phagocyte adherence (Carvalho *et al.*, 2004) to the connective tissue. This may, therefore, constitute an important incentive to infected phagocytes remaining or disseminating to the skin of dogs during different phases of visceral leishmaniasis. A pattern of parasite dissemination to inflammatory sites has been confirmed in an experimental model of cutaneous leishmaniasis (Bertho *et al.*, 1994).

In fact, it is not well established how *Leishmania* parasites are transported from the original infection site in the skin to other organs. Transport of amastigotes by cutaneous Langerhans cells from the skin to the draining lymph nodes was demonstrated by Moll and collaborators (1993) in murine models of infection, and Fiorini and collaborators (2002)

detected myeloid cells containing *Leishmania* in human blood (Fiorini *et al.*, 2002). In all lesions caused by *Leishmania*, the parasite is found inside mononuclear phagocytes, which maintain close contact with the extracellular matrix and cells of the connective tissue (Abreu-Silva *et al.*, 2004). In only a few instances have parasites been found free in the tissues (Santos-Gomes *et al.*, 2000; Wilson *et al.*, 1987). These data suggest that the main interface between *Leishmania* and the host may be the mononuclear phagocyte cell surface. It also suggests that the relevant information for *Leishmania* to remain within or to leave tissues may be expressed on the surface of these phagocytes.

Another interesting aspect of cell migration that affects the tissue in leishmaniasis is related to the loss of lymphoid tissue structure in the spleen, as reported by some authors for visceral leishmaniasis. Such alterations have been described in human beings by Veress and collaborators in 1977 (Veress *et al.*, 1977), and have recently been re-examined in a number of important studies performed by the group led by Paul Kaye. These studies have shown that the observed changes in the lymphoid tissue of the spleen are due to impaired leukocyte migration into the white pulp induced by TNF (Engwerda *et al.*, 2002) and IL10 (Ato *et al.*, 2002). Basically, a number of interactions between lymphocytes and mononuclear phagocytes may be disrupted in the marginal zone of the spleen, disturbing the entry of cells into the white pulp and follicle organization (Ato *et al.*, 2002; Engwerda *et al.*, 2002). We recently observed a similar pattern of lymphoid tissue disorganization in the spleens of dogs with visceral leishmaniasis. Such changes were more prominent in animals with a susceptibility pattern in response to *L. chagasi* infection (negative leishmanin skin test and positive spleen culture for *Leishmania*) than in non-infected animals or in animals with a positive leishmanin skin test (Santana *et al.*, 2008). These alterations of the white pulp appear to be associated with the disappearance of a population of marginal zone macrophages defined by an H11 monoclonal antibody staining (Aguiar *et al.*, 2004). Whether this represents a cause or consequence of tissue disorganization remains unclear. Such a loss of lymphoid tissue structure may, however, underlie the increased susceptibility of these animals to bacterial infection and enhanced dissemination of *Leishmania* during late stages of the disease.

Recently, the cellular response in spleen was investigated (Lages *et al.*, 2008; Strauss-Ayali *et al.*, 2007). In both works, the mRNA expression levels for a wide panel of cytokines, transcription factors, and chemokines were examined. Both studies clearly show that Th1-1 and Th-2 immune responses occur simultaneously in the spleen during canine *L. infantum* infection (Lage *et al.*, 2007; Strauss-Ayali *et al.*, 2007). The frequency of IL-12 and IFN- γ expression within symptomatic dogs was significantly different from that of the uninfected group, although there were no significant differences between the symptomatic groups with respect to the expression of these cytokines (Lage *et al.*, 2007). In accordance, the other study identified higher IFN- γ , T-bet, IP-10, and RANTES mRNA levels in infected dogs during both oligosymptomatic and polysymptomatic stages of the disease (Strauss-Ayali *et al.*, 2007). These results agree with those reported by Quinnell and collaborators (Quinnell *et al.*, 2001b), who suggested that IFN- γ expression is not an appropriate indicator of resistance since asymptomatic and polysymptomatic dogs accumulated similar levels of this cytokine in tissues, as is the case in humans, mice, and hamsters (Lage *et al.*, 2007). On the other hand, the Th-2 immune response in dogs was differentially described by these works. In one study, positive correlations between the levels of IL-10 expression with respect to the progression of the disease were observed. The other authors identified increased IL-4 and IL-5 expression

during oligosymptomatic disease instead of enhanced expression levels of the Treg (T regulatory)-associated cytokines, IL-10 and TGF- β (Strauss-Ayali *et al.*, 2007).

Blood Compartment

The results of the serological analysis of VL-infected dogs also identified mixed Th-1 and Th-2 responses in the serum of infected dogs, with detectable expression levels of IFN- γ , TNF- α , and IL-12 together with IL-4 and IL-10. However, when clinical indications are considered alongside the biochemical data, the Th-2 response appears to be predominant, since the expression of IL-4 increased within the symptomatic group while the expression of IL-12 increased within the asymptomatic group (Santos-Gomes *et al.*, 2002).

The nature of the dog's PBMC responses to *Leishmania* is not completely understood. Asymptomatic dogs show protective immunity, which has generally been associated with a strong proliferative response of peripheral blood lymphocytes to leishmanial antigens (Cabral *et al.*, 1992; Pinelli *et al.*, 1995; Pinelli *et al.*, 1994). However, development of a Th-1 and Th-2 mixed response by antigen-stimulated PBMCs from asymptomatic dogs expressing IL-2, IFN- γ , and IL-10 mRNA transcripts has also been reported. Although, in these studies, IL-2 and IFN- γ predominated in asymptomatic dogs, the development of symptomatic infections could not be related to IL-10 expression (Carvalho *et al.*, 1994; Chamizo *et al.*, 2005). Thus, in contrast to what occurs in human visceral leishmaniasis, the role played by PBMC-expressed IL-10 in *L. chagasi*-infected dogs is not well established (Carvalho *et al.*, 1994).

Dogs with symptomatic CVL (Berrahal *et al.*, 1996) present with depressed T cell-mediated functions and high levels of specific antibodies (Abranches *et al.*, 1991; Barbieri, 2006; Killick-Kendrick *et al.*, 1994; Oliva *et al.*, 2004; Santos-Gomes *et al.*, 2002). These animals present immunological changes involving T cells, including absence of delayed type hypersensitivity (DTH) to *Leishmania* antigens (Berrahal *et al.*, 1996; Oliva *et al.*, 2004; Quinnell *et al.*, 2001b), decreased T cell numbers in the peripheral blood (Cabral *et al.*, 1998; Killick-Kendrick *et al.*, 1994; Oliva *et al.*, 2004), and absence of IFN- γ and IL-2 production by PBMCs *in vitro* (Alvar *et al.*, 2004; Oliva *et al.*, 2004; Pinelli *et al.*, 1994). Interestingly, the Th-1 cytokine profile in bone marrow aspirates positively correlates with humoral, but not with lymphoproliferative responses to *Leishmania* antigen. It is noteworthy that increased accumulation of IL-4, IL-10, and IL-18 mRNA was not observed in infected dogs, and the mRNA for these cytokines did not correlate with antibody or proliferative responses. However, infected dogs with detectable IL-4 mRNA display significantly more severe symptoms (Quinnell *et al.*, 2001b). These data suggest that clinical symptoms are not due to a deficiency in IFN- γ production.

It is well established that early events are considered to be a determinant of infection outcome in humans and mice (Gomes *et al.*, 2000; Rogers and Titus, 2004; Shankar and Titus, 1993; Veras *et al.*, 2006). Prediction of dog immune responses *in vivo* early after exposure to *L. chagasi* is a difficult task (human models). We have established an *in vitro* priming system (PIV) using naïve canine PBMCs in order to assess dog PIV immune response to *L. chagasi* (Rodrigues *et al.*, 2008). We co-cultivated PBMCs primarily stimulated with *L. chagasi* *in vitro* with autologous infected macrophages and found that IFN- γ mRNA is upregulated in these cells compared to control unstimulated cells. IL-4 and IL-10 mRNA expression in *L.*

chagasi-stimulated PBMCs was similar to control unstimulated PBMCs when incubated with infected macrophages. Surprisingly, correlation studies showed that a lower IFN- γ /IL-4 expression ratio correlates with a lower percentage of infection. We proposed that the direct correlation between the IFN- γ /IL-4 ratio and parasite load is dependent on the positive correlation of both IFN- γ and IL-4 expression with lower parasite infection. This PIV system was shown to be useful in evaluating the dog immune response to *L. chagasi*, and the results indicate that a balanced expression of IFN- γ and IL-4 by these naïve cells is associated with control of parasite infection *in vitro* (Rodrigues *et al.*, 2008).

In experimental infections, intradermal inoculation of promastigotes triggers asymptomatic infections, and PBMCs from these dogs stimulated with soluble leishmanial antigens (SLA) *in vitro* express both Th-1 cytokines, such as IL-12, IFN- γ , TNF- α , and IL-18, and Th-2 cytokines, such as IL-4, IL-6, and IL-10. Despite the fact that PBMCs from these asymptomatic dogs present such apparently mixed Th-1 and Th-2 responses, they predominantly produce IL-12 and IFN- γ . In accordance with a previous observation (Pinelli *et al.*, 1994), these data support the protective immune response observed in these animals (Chamizo *et al.*, 2005). We recently observed that PBMCs from immunized dogs and then subcutaneously challenged with *L. chagasi* promastigotes are still asymptomatic. PBMCs from these apparently protected dogs liberate IFN- γ into the cell supernatant upon *L. chagasi* stimulation *in vitro* (Rodrigues *et al.*, 2007). Moreover these cells express IFN- γ but not IL-4 mRNA (Rodrigues *et al.*, 2007), showing that these dogs display a predominant Th-1 type of immune response. On the other hand, dogs experimentally infected by intravenous inoculation of amastigotes develop progressive symptomatic infections. PBMCs from these dogs produce reduced levels of both Th-1 and Th-2 cytokines (IFN- γ , IL-2, IL-12, IL-6, and IL-10) during the active phase of the disease (Santos-Gomes *et al.*, 2002).

In murine models, it is well established that macrophages participate in parasite killing via reactive oxygen and nitrogen intermediate-dependent mechanisms. However, mechanisms involved in *Leishmania* killing by canine macrophages have not been as thoroughly investigated. There are cumulative data implicating canine macrophages in parasite killing by a NO-dependent mechanism. NO produced by macrophages has been found to be the principal effector molecule responsible for mediating intracellular killing of *Leishmania* (Holzmüller *et al.*, 2006; Panaro *et al.*, 2001; Pinelli *et al.*, 2000). A canine macrophage cell line incubated with supernatant (containing IFN- γ , IL-2, and TNF- α) produced significant amounts of NO, sufficient to mediate *L. infantum*-killing (Pinelli *et al.*, 2000). PBMCs from vaccinated dogs were also able to reduce macrophage infection via an NO-dependent mechanism upon *in vitro* stimulation with both *Leishmania* promastigotes and concanavalin A (ConA). This effect was potentiated by the addition of LPS (Panaro *et al.*, 2001). Using a macrophage cell line, Pinelli and collaborators (Pinelli *et al.*, 2000) showed that parasite burden is reduced upon activation of cells with cytokine-rich supernatants. These supernatants were obtained from a *Leishmania*-specific T cell-line generated from dogs immunized with soluble Ag (Panaro *et al.*, 2001). Infected canine macrophages incubated with autologous lymphocytes of immunized dogs also induced IFN- γ with increased NO production (Holzmüller *et al.*, 2005). The increased IFN- γ production and NO release by macrophages suggest a role for this cytokine in iNOS induction. We recently established an *in vitro* model to test whether PBMC supernatants from asymptomatic dogs immunized with promastigote lysates and infected with *L. chagasi* promastigotes were able to stimulate PBMC-derived macrophages from healthy dogs to

control parasite infection (Rodrigues *et al.*, 2007). Using our system, we demonstrated for the first time that PBMCs from these asymptomatic dogs stimulated exclusively with *L. chagasi* *in vitro* reduce macrophage infection by the parasite (Rodrigues *et al.*, 2007). Moreover this effect is associated with high IFN- γ , but not IL-4, mRNA expression and release of this Th1 cytokine into the PBMC supernatant via an NO-dependent mechanism, as AMG reversed this effect (Rodrigues *et al.*, 2007). In contrast to other works (Panaro *et al.*, 2001), PBMCs in our system were exclusively stimulated with *L. chagasi* *in vitro*. Additionally, the protective response of these dogs to *L. chagasi* was demonstrated by the positive proliferative response to *Leishmania* antigens exhibited by PBMCs from these dogs *in vitro* (Rodrigues *et al.*, 2007). In addition, PBMCs from the majority of these immunized and experimentally infected dogs expressed IFN- γ mRNA and released IFN- γ upon LSA stimulation. These data suggest that lymphocytes from apparently protected dogs produce cytokines related to a protective immune response (Rodrigues *et al.*, 2007). A recent study demonstrated that, although Th-1 and Th-2 cytokines are produced in asymptomatic *Leishmania*-infected dogs, there is a prevalent Th-1 cytokine response that confers immunity against the parasite (Chamizo *et al.*, 2005). Finally, our data reinforce the notion that the leishmanicidal effect of canine macrophages is NO-dependent (Rodrigues *et al.*, 2007).

It was recently demonstrated that there are some differences in iNOS expression in lesion macrophages *in situ*. iNOS-negative dermal and splenic macrophages contain numerous *Leishmania* amastigotes. In contrast, dermal and splenic macrophages, which present high iNOS expression, contain few or no amastigotes, suggesting that iNOS-positive activated macrophages are able to destroy and/or do not allow multiplication of intracellular amastigotes (Zafra *et al.*, 2008). PBMC-derived macrophages infected with *L. infantum* produce a significantly higher amount of NO than uninfected macrophages *in vitro* (Panaro *et al.*, 2008; Rodrigues *et al.*, 2007). In a comparison between infected dogs, the levels of NO in supernatants of *Leishmania*-infected macrophages were significantly higher in symptomatic than in asymptomatic animals. However, four months after diagnosis, the addition of autologous lymphocytes significantly decreased NO production only in symptomatic dogs, while NO production by macrophages co-cultured with autologous lymphocytes was significantly reduced eight months after diagnosis in *Leishmania*-infected macrophages from both asymptomatic and symptomatic dogs (Panaro *et al.*, 2008). These higher levels of NO observed during follow-up of symptom-free (only 8 months) animals may suggest a protective role for this molecule in long-term asymptomatic parasitism.

CONCLUSION

The nature of the dog's cellular immune response is not completely understood. Evidence points that although CVL dogs develop a mixed Th-1 and Th-2 cellular immune response, asymptomatic dogs present positive lymphoproliferative assay *in vitro* or/and a positive skin test early in infection, as well as predominance of Th-1 cytokines. On the other hand, as the disease progresses in susceptible dogs, the protective responses diminish with involvement of either IL-4 or IL-10 in uncontrolled infection. Disease progression occurs together with parasite dissemination. *Leishmania*-infected mononuclear phagocyte may stay or leave inflammatory sites disseminating the parasite through the host tissues. The migration of these

cells depends upon the leukocyte phenotype and is modulated by parasite burden that results in changes in integrin function and in the expression of chemokine receptors.

ACKNOWLEDGMENTS

We thank all of our collaborators from the Immunology and Bio-Intervention Laboratory at the Gonalo Moniz Research Center, FIOCRUZ, Bahia, Brazil. This work was supported by the Bahia State Research Supporting Agency - FAPESB and The Conselho Nacional de Desenvolvimento Científico e Tecnológico (The Brazilian National Research Council).

REFERENCES

- Abranches, P., Silva-Pereira, M.C., Conceicao-Silva, F.M., Santos-Gomes, G.M., Janz, J.G., 1991, Canine leishmaniasis: pathological and ecological factors influencing transmission of infection. *The Journal of Parasitology* 77, 557-561.
- Abreu-Silva, A.L., Calabrese, K.S., Mortara, R.A., Tedesco, R.C., Cardoso, F.O., Carvalho, L.O., Goncalves da Costa, S.C., 2004, Extracellular matrix alterations in experimental murine *Leishmania (L.) amazonensis* infection. *Parasitology* 128, 385-390.
- Acedo-Sanchez, C., Morillas-Marquez, F., Sanchiz-Marin, M.C., Martin-Sanchez, J., 1998, Changes in antibody titres against *Leishmania infantum* in naturally infected dogs in southern Spain. *Veterinary Parasitology* 75, 1-8.
- Aguiar, P.H., Borges dos Santos, R.R., Lima, C.A., Rios de Sousa Gomes, H., Larangeira, D.F., Santos, P.M., Barrouin-Melo, S.M., Conrado dos-Santos, W.L., Pontes-de-Carvalho, L., 2004, Production of monoclonal antibodies against canine leukocytes. *Hybrid Hybridomics* 23, 127-132.
- Alvar, J., Canavate, C., Molina, R., Moreno, J., Nieto, J., 2004, Canine leishmaniasis. *Advances in Parasitology* 57, 1-88.
- Alvar, J., Molina, R., San Andres, M., Tesouro, M., Nieto, J., Vitutia, M., Gonzalez, F., San Andres, M.D., Boggio, J., Rodriguez, F., *et al.*, 1994, Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. *Annals of Tropical Medicine and Parasitology* 88, 371-378.
- Ashford, D.A., David, J.R., Freire, M., David, R., Sherlock, I., Eulalio, M.C., Sampaio, D.P., Badaro, R., 1998, Studies on control of visceral leishmaniasis: impact of dog control on canine and human visceral leishmaniasis in Jacobina, Bahia, Brazil. *The American Journal of Tropical Medicine and Hygiene* 59, 53-57.
- Ato, M., Stager, S., Engwerda, C.R., Kaye, P.M., 2002, Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. *Nature Immunology* 3, 1185-1191.
- Baneth, G., Aroch, I., 2008, Canine leishmaniasis: a diagnostic and clinical challenge. *Veterinary Journal* 175, 14-15.
- Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008, Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. *Trends in Parasitology* 24, 324-330.

- Barbieri, C.L., 2006, Immunology of canine leishmaniasis. *Parasite Immunology* 28, 329-337.
- Barrouin-Melo, S.M., Larangeira, D.F., Trigo, J., Aguiar, P.H., dos-Santos, W.L., Pontes-de-Carvalho, L., 2004, Comparison between splenic and lymph node aspirations as sampling methods for the parasitological detection of *Leishmania chagasi* infection in dogs. *Memorias do Instituto Oswaldo Cruz* 99, 195-197.
- Berrahal, F., Mary, C., Roze, M., Berenger, A., Escoffier, K., Lamouroux, D., Dunan, S., 1996, Canine leishmaniasis: identification of asymptomatic carriers by polymerase chain reaction and immunoblotting. *The American Journal of Tropical Medicine and Hygiene* 55, 273-277.
- Bertho, A.L., Santiago, M.A., Coutinho, S.G., 1994, An experimental model of the production of metastases in murine cutaneous leishmaniasis. *The Journal of Parasitology* 80, 93-99.
- Bettini, S., Gramiccia, M., Gradoni, L., Atzeni, M.C., 1986, Leishmaniasis in Sardinia: II. Natural infection of *Phlebotomus perniciosus* Newstead, 1911, by *Leishmania infantum* Nicolle, 1908, in the province of Cagliari. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 80, 458-459.
- Blackwell, J.M., Goswami, T., Evans, C.A., Sibthorpe, D., Papo, N., White, J.K., Searle, S., Miller, E.N., Peacock, C.S., Mohammed, H., Ibrahim, M., 2001, SLC11A1 (formerly NRAMPI) and disease resistance. *Cellular Microbiology* 3, 773-784.
- Bodman-Smith, K.B., Mbuchi, M., Culley, F.J., Bates, P.A., Raynes, J.G., 2002, C-reactive protein-mediated phagocytosis of *Leishmania donovani* promastigotes does not alter parasite survival or macrophage responses. *Parasite Immunology* 24, 447-454.
- Brachelente, C., Muller, N., Doherr, M.G., Sattler, U., Welle, M., 2005, Cutaneous leishmaniasis in naturally infected dogs is associated with a T helper-2-biased immune response. *Veterinary Pathology* 42, 166-175.
- Buates, S., Matlashewski, G., 2001, General suppression of macrophage gene expression during *Leishmania donovani* infection. *Journal of Immunology* 166, 3416-3422.
- Bueno, R., Mello, M.N., Menezes, C.A., Dutra, W.O., Santos, R.L., 2005, Phenotypic, functional, and quantitative characterization of canine peripheral blood monocyte-derived macrophages. *Memorias do Instituto Oswaldo Cruz* 100, 521-524.
- Burattini, M.N., Coutinho, F.A.B., Lopes, L.F., Massad, E., 1995, Modelling of dynamics of Leishmaniasis considering human, animal host and vector populations. *Mathematical Modelling and Analyses of Scientific Computing* 26, 1-16.
- Cabral, M., O'Grady, J., Alexander, J., 1992, Demonstration of *Leishmania* specific cell mediated and humoral immunity in asymptomatic dogs. *Parasite Immunology* 14, 531-539.
- Cabral, M., O'Grady, J.E., Gomes, S., Sousa, J.C., Thompson, H., Alexander, J., 1998, The immunology of canine leishmaniasis: strong evidence for a developing disease spectrum from asymptomatic dogs. *Veterinary Parasitology* 76, 173-180.
- Cardoso, L., Neto, F., Sousa, J.C., Rodrigues, M., Cabral, M., 1998, Use of a leishmanin skin test in the detection of canine *Leishmania*-specific cellular immunity. *Veterinary Parasitology* 79, 213-220.
- Carlos, T.M., Harlan, J.M., 1994, Leukocyte-endothelial adhesion molecules. *Blood* 84, 2068-2101.
- Carvalho, D.G., Barbosa, A., Jr., D'El-Rei Hermida, M., Clarencio, J., Pinheiro, N.F., Jr., Veras, P.S.T., dos-Santos, W.L., 2004, The modelling of mononuclear phagocyte-

- connective tissue adhesion in vitro: application to disclose a specific inhibitory effect of *Leishmania* infection. *Experimental Parasitology* 107, 189-199.
- Carvalho, E.M., Bacellar, O., Brownell, C., Regis, T., Coffman, R.L., Reed, S.G., 1994, Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. *Journal of Immunology* 152, 5949-5956.
- Chamizo, C., Moreno, J., Alvar, J., 2005, Semi-quantitative analysis of cytokine expression in asymptomatic canine leishmaniasis. *Veterinary Immunology and Immunopathology* 103, 67-75.
- Courtenay, O., Quinnell, R.J., Garcez, L.M., Shaw, J.J., Dye, C., 2002, Infectiousness in a cohort of brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. *The Journal of Infectious Diseases* 186, 1314-1320.
- De Luna, R., Ferrante, M., Severino, L., Ambrosio, R., Piantedosi, D., Gradoni, L., Lucisano, A., Persechino, A., 2000, Decreased lipid fluidity of the erythrocyte membrane in dogs with leishmaniasis-associated anaemia. *Journal of Comparative Pathology* 122, 213-216.
- Desjeux, P., 2001a, The increased risk factors for leishmaniasis worldwide. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95, 239-243.
- Desjeux, P., 2001b, Worldwide increasing risk factors for leishmaniasis. *Medical Microbiology and Immunology* 190, 77-79.
- Desjeux, P., 2004, Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases* 27, 305-318.
- dos-Santos, W.L., David, J., Badaro, R., de-Freitas, L.A., 2004, Association between skin parasitism and a granulomatous inflammatory pattern in canine visceral leishmaniosis. *Parasitology Research* 92, 89-94.
- Dye, C., 1996, The logic of visceral leishmaniasis control. *The American Journal of Tropical Medicine and Hygiene* 55, 125-130.
- Engwerda, C.R., Ato, M., Cotterell, S.E., Mynott, T.L., Tschannerl, A., Gorak-Stolinska, P.M., Kaye, P.M., 2002, A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during *Leishmania donovani* infection. *The American Journal of Pathology* 161, 429-437.
- Fiorini, M., Messina, M.F., Barracchia, A., 2002, Peripheral intramonocytic and intraneutrophil leishmanias observed in a chronic myelomonocytic leukemia (CMMoL) patient. *Haematologica* 87, E1M21.
- Fisa, R., Gallego, M., Castillejo, S., Aisa, M.J., Serra, T., Riera, C., Carrio, J., Gallego, J., Portus, M., 1999, Epidemiology of canine leishmaniosis in Catalonia (Spain) the example of the Priorat focus. *Veterinary Parasitology* 83, 87-97.
- Giunchetti, R.C., Correa-Oliveira, R., Martins-Filho, O.A., Teixeira-Carvalho, A., Roatt, B.M., de Oliveira Aguiar-Soares, R.D., de Souza, J.V., das Dores Moreira, N., Malaquias, L.C., Mota e Castro, L.L., de Lana, M., Reis, A.B., 2007, Immunogenicity of a killed *Leishmania* vaccine with saponin adjuvant in dogs. *Vaccine* 25, 7674-7686.
- Giunchetti, R.C., Martins-Filho, O.A., Carneiro, C.M., Mayrink, W., Marques, M.J., Tafuri, W.L., Correa-Oliveira, R., Reis, A.B., 2008a, Histopathology, parasite density and cell phenotypes of the popliteal lymph node in canine visceral leishmaniasis. *Veterinary Immunology and Immunopathology* 121, 23-33.
- Giunchetti, R.C., Mayrink, W., Carneiro, C.M., Correa-Oliveira, R., Martins-Filho, O.A., Marques, M.J., Tafuri, W.L., Reis, A.B., 2008b, Histopathological and immunohistochemical investigations of the hepatic compartment associated with

- parasitism and serum biochemical changes in canine visceral leishmaniasis. *Research in Veterinary Science* 84, 269-277.
- Gomes, N.A., Barreto-de-Souza, V., DosReis, G.A., 2000, Early in vitro priming of distinct T(h) cell subsets determines polarized growth of visceralizing *Leishmania* in macrophages. *International Immunology* 12, 1227-1233.
- Goncalves, R., Vieira, E.R., Melo, M.N., Gollob, K.J., Mosser, D.M., Tafuri, W.L., 2005, A sensitive flow cytometric methodology for studying the binding of *L. chagasi* to canine peritoneal macrophages. *BMC of Infectious Disease* 5, 39.
- Gruenheid, S., Pinner, E., Desjardins, M., Gros, P., 1997, Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. *Journal of Experimental Medicine* 185, 717-730.
- Holzmuller, P., Bras-Goncalves, R., Lemesre, J.L., 2006, Phenotypical characteristics, biochemical pathways, molecular targets and putative role of nitric oxide-mediated programmed cell death in *Leishmania*. *Parasitology* 132 Suppl, S19-32.
- Holzmuller, P., Cavaleyra, M., Moreaux, J., Kovacic, R., Vincendeau, P., Papierok, G., Lemesre, J.L., 2005, Lymphocytes of dogs immunised with purified excreted-secreted antigens of *Leishmania infantum* co-incubated with *Leishmania* infected macrophages produce IFN gamma resulting in nitric oxide-mediated amastigote apoptosis. *Veterinary Immunology and Immunopathology* 106, 247-257.
- Killick-Kendrick, R., Killick-Kendrick, M., Pinelli, E., Del Real, G., Molina, R., Vitutia, M.M., Canavate, M.C., Nieto, J., 1994, A laboratory model of canine leishmaniasis: the inoculation of dogs with *Leishmania infantum* promastigotes from midguts of experimentally infected phlebotomine sandflies. *Parasite* (Paris, France) 1, 311-318.
- Lage, R.S., Oliveira, G.C., Busek, S.U., Guerra, L.L., Giunchetti, R.C., Correa-Oliveira, R., Reis, A.B., 2007, Analysis of the cytokine profile in spleen cells from dogs naturally infected by *Leishmania chagasi*. *Veterinary Immunology and Immunopathology* 115, 135-145.
- Lages, C.S., Suffia, I., Velilla, P.A., Huang, B., Warshaw, G., Hildeman, D.A., Belkaid, Y., Chougnet, C., 2008, Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. *Journal of Immunology* 181, 1835-1848.
- Leontides, L.S., Saridomichelakis, M.N., Billinis, C., Kontos, V., Koutinas, A.F., Galatos, A.D., Mylonakis, M.E., 2002, A cross-sectional study of *Leishmania* spp. infection in clinically healthy dogs with polymerase chain reaction and serology in Greece. *Veterinary Parasitology* 109, 19-27.
- Martinez-Moreno, A., Moreno, T., Martinez-Moreno, F.J., Acosta, I., Hernandez, S., 1995, Humoral and cell-mediated immunity in natural and experimental canine leishmaniasis. *Veterinary Immunology and Immunopathology* 48, 209-220.
- Mauricio, I., Campino, L., Abranches, P., 1995, [Quality control of the micro-ELISA technic applied to the diagnosis of human and canine visceral leishmaniasis]. *Acta Medica Portuguesa* 8, 607-611.
- Mauricio, I.L., Stothard, J.R., Miles, M.A., 2000, The strange case of *Leishmania chagasi*. *Parasitology Today* 16, 188-189.
- Molina, R., Amela, C., Nieto, J., San-Andres, M., Gonzalez, F., Castillo, J.A., Lucientes, J., Alvar, J., 1994, Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 88, 491-493.

- Moll, H., Fuchs, H., Blank, C., Rollinghoff, M., 1993, Langerhans cells transport *Leishmania major* from the infected skin to the draining lymph node for presentation to antigen-specific T cells. *European Journal of Immunology* 23, 1595-1601.
- Moreno, J., Alvar, J., 2002, Canine leishmaniasis: epidemiological risk and the experimental model. *Trends in Parasitology* 18, 399-405.
- Murray, H.W., 2001, Tissue granuloma structure-function in experimental visceral leishmaniasis. *International Journal of Experimental Pathology* 82, 249-267.
- Oliva, G., Foglia Manzillo, V., Pagano, A., 2004, [Canine leishmaniasis: evolution of the chemotherapeutic protocols]. *Parassitologia* 46, 231-234.
- Palatnik-de-Sousa, C.B., dos Santos, W.R., Franca-Silva, J.C., da Costa, R.T., Reis, A.B., Palatnik, M., Mayrink, W., Genaro, O., 2001, Impact of canine control on the epidemiology of canine and human visceral leishmaniasis in Brazil. *The American journal of Tropical Medicine and Hygiene* 65, 510-517.
- Panaro, M.A., Acquafredda, A., Lisi, S., Lofrumento, D.D., Mitolo, V., Sisto, M., Fasanella, A., Trotta, T., Bertani, F., Consenti, B., Brandonisio, O., 2001, Nitric oxide production by macrophages of dogs vaccinated with killed *Leishmania infantum* promastigotes. *Comparative Immunology, Microbiology and Infectious Diseases* 24, 187-195.
- Panaro, M.A., Brandonisio, O., de Caprariis, D., Cavallo, P., Cianciulli, A., Mitolo, V., Otranto, D., 2008, Canine leishmaniasis in Southern Italy: a role for nitric oxide released from activated macrophages in asymptomatic infection? *Parasites and vectors* 1, 10.
- Pinelli, E., Gebhard, D., Mommaas, A.M., van Hoeij, M., Langermans, J.A., Ruitenbergh, E.J., Rutten, V.P., 2000, Infection of a canine macrophage cell line with *Leishmania infantum*: determination of nitric oxide production and anti-leishmanial activity. *Veterinary Parasitology* 92, 181-189.
- Pinelli, E., Gonzalo, R.M., Boog, C.J., Rutten, V.P., Gebhard, D., del Real, G., Ruitenbergh, E.J., 1995, *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. *European Journal of Immunology* 25, 1594-1600.
- Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Real, G., Ruitenbergh, J., 1994, Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. *Infection and Immunity* 62, 229-235.
- Pinelli, E., van der Kaaij, S.Y., Slappendel, R., Fragio, C., Ruitenbergh, E.J., Bernadina, W., Rutten, V.P., 1999, Detection of canine cytokine gene expression by reverse transcription-polymerase chain reaction. *Veterinary Immunology and Immunopathology* 69, 121-126.
- Pinheiro, N.F., Jr., Hermida, M.D., Macedo, M.P., Mengel, J., Bafica, A., dos-Santos, W.L., 2006, *Leishmania* infection impairs beta 1-integrin function and chemokine receptor expression in mononuclear phagocytes. *Infection and Immunity* 74, 3912-3921.
- Pucheu-Haston, C.M., Shuster, D., Olivry, T., Brianceau, P., Lockwood, P., McClanahan, T., de Waal Malefyt, R., Mattson, J.D., Hammerberg, B., 2006, A canine model of cutaneous late-phase reactions: prednisolone inhibition of cellular and cytokine responses. *Immunology* 117, 177-187.
- Queiroz, P.V., Monteiro, G.R., Macedo, V.P., Rocha, M.A., Batista, L.M., Queiroz, J.W., Jeronimo, S.M., Ximenes, M.F., 2008, Canine visceral leishmaniasis in urban and rural areas of Northeast Brazil. *Research in Veterinary Science*.

- Quinnell, R.J., Courtenay, O., Davidson, S., Garcez, L., Lambson, B., Ramos, P., Shaw, J.J., Shaw, M.A., Dye, C., 2001a, Detection of *Leishmania infantum* by PCR, serology and cellular immune response in a cohort study of Brazilian dogs. *Parasitology* 122, 253-261.
- Quinnell, R.J., Courtenay, O., Shaw, M.A., Day, M.J., Garcez, L.M., Dye, C., Kaye, P.M., 2001b, Tissue cytokine responses in canine visceral leishmaniasis. *The Journal of Infectious Diseases* 183, 1421-1424.
- Reis, A.B., Teixeira-Carvalho, A., Vale, A.M., Marques, M.J., Giunchetti, R.C., Mayrink, W., Guerra, L.L., Andrade, R.A., Correa-Oliveira, R., Martins-Filho, O.A., 2006, Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*. *Veterinary Immunology and Immunopathology* 112, 102-116.
- Rodrigues, C.A., Batista, L.F., Filho, R.S., Santos, C.D., Pinheiro, C.G., Almeida, T.F., Freitas, L.A., Veras, P.S., 2008, IFN-gamma expression is up-regulated by peripheral blood mononuclear cells (PBMC) from non-exposed dogs upon *Leishmania chagasi* promastigote stimulation *in vitro*. *Veterinary Immunology and Immunopathology*.
- Rodrigues, C.A., Batista, L.F., Teixeira, M.C., Pereira, A.M., Santos, P.O., de Sa Oliveira, G.G., de Freitas, L.A., Veras, P.S., 2007, Peripheral blood mononuclear cell supernatants from asymptomatic dogs immunized and experimentally challenged with *Leishmania chagasi* can stimulate canine macrophages to reduce infection *in vitro*. *Veterinary Parasitology* 143, 197-205.
- Rogers, K.A., Titus, R.G., 2004, Characterization of the early cellular immune response to *Leishmania major* using peripheral blood mononuclear cells from *Leishmania*-naive humans. *The American Journal of Tropical Medicine and Hygiene* 71, 568-576.
- Rogers, M.E., Ilg, T., Nikolaev, A.V., Ferguson, M.A., Bates, P.A., 2004, Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. *Nature* 430, 463-467.
- Sampaio, W.M., Moura, E.P., Arruda, F.C., Ribeiro, R.R., Alves, C.F., Melo, F.A., Fernandes, A.P., Michalick, M.S., Melo, M.N., Tafuri, W.L., Tafuri, W.L., 2007, *In vitro* binding and survival assays of *Leishmania* parasites to peripheral blood monocytes and monocyte-derived macrophages isolated from dogs naturally and experimentally infected with *Leishmania (Leishmania) chagasi*. *BMC Veterinary Research* 3, 11.
- Sanchez-Robert, E., Altet, L., Sanchez, A., Francino, O., 2005, Polymorphism of *Slc11a1* (*Nramp1*) gene and canine leishmaniasis in a case-control study. *The Journal of Heredity* 96, 755-758.
- Sanchez-Robert, E., Altet, L., Utzet-Sadurni, M., Giger, U., Sanchez, A., Francino, O., 2008, *Slc11a1* (formerly *Nramp1*) and susceptibility to canine visceral leishmaniasis. *Veterinary Research* 39, 36.
- Santana, C.C., Vassallo, J., de Freitas, L.A., Oliveira, G.G., Pontes-de-Carvalho, L.C., dos-Santos, W.L., 2008, Inflammation and structural changes of splenic lymphoid tissue in visceral leishmaniasis: a study on naturally infected dogs. *Parasite Immunology* 30, 515-524.
- Santos-Gomes, G.M., Campino, L., Abranches, P., 2000, Canine experimental infection: intradermal inoculation of *Leishmania infantum* promastigotes. *Memorias do Instituto Oswaldo Cruz* 95, 193-198.

- Santos-Gomes, G.M., Rosa, R., Leandro, C., Cortes, S., Romao, P., Silveira, H., 2002, Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. *Veterinary Immunology and Immunopathology* 88, 21-30.
- Shankar, A.H., Titus, R.G., 1993, *Leishmania major*-specific, CD4+, major histocompatibility complex class II-restricted T cells derived in vitro from lymphoid tissues of naive mice. *Journal of Experimental Medicine* 178, 101-111.
- Solano-Gallego, L., Fernandez-Bellon, H., Morell, P., Fondevila, D., Alberola, J., Ramis, A., Ferrer, L., 2004, Histological and immunohistochemical study of clinically normal skin of *Leishmania infantum*-infected dogs. *Journal of Comparative Pathology* 130, 7-12.
- Solano-Gallego, L., Llull, J., Ramos, G., Riera, C., Arboix, M., Alberola, J., Ferrer, L., 2000, The Ibizian hound presents a predominantly cellular immune response against natural *Leishmania* infection. *Veterinary Parasitology* 90, 37-45.
- Solano-Gallego, L., Morell, P., Arboix, M., Alberola, J., Ferrer, L., 2001, Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. *Journal of Clinical Microbiology* 39, 560-563.
- Solano-Gallego, L., Rodriguez-Cortes, A., Trotta, M., Zampieron, C., Razia, L., Furlanello, T., Caldin, M., Roura, X., Alberola, J., 2007, Detection of *Leishmania infantum* DNA by fret-based real-time PCR in urine from dogs with natural clinical leishmaniosis. *Veterinary Parasitology* 147, 315-319.
- Steigerwald, M., Moll, H., 2005, *Leishmania major* modulates chemokine and chemokine receptor expression by dendritic cells and affects their migratory capacity. *Infection and Immunity* 73, 2564-2567.
- Strauss-Ayali, D., Baneth, G., Jaffe, C.L., 2007, Splenic immune responses during canine visceral leishmaniasis. *Veterinary Research* 38, 547-564.
- Veras, P.S., Welby-Borges, M., de Santana, C.D., Nihei, J., Cardillo, F., de Freitas, L.A., 2006, *Leishmania amazonensis*: participation of regulatory T and B cells in the *in vitro* priming (PIV) of CBA/J spleen cells susceptible response. *Experimental Parasitology* 113, 201-205.
- Veress, B., Omer, A., Satir, A.A., El Hassan, A.M., 1977, Morphology of the spleen and lymph nodes in fatal visceral leishmaniasis. *Immunology* 33, 605-610.
- World Health Organization 1999. Leishmaniasis. Fourteenth Programme Report. UNDP/World Bank/Special Programme for Research and Training in Tropical Disease. In *Tropical Disease Research Progress 1997-1998* (Geneva), pp. 1-6.
- Wilson, M.E., Innes, D.J., Sousa, A.D., Pearson, R.D., 1987, Early histopathology of experimental infection with *Leishmania donovani* in hamsters. *The Journal of Parasitology* 73, 55-63.
- Zafra, R., Jaber, J.R., Perez-Ecija, R.A., Barragan, A., Martinez-Moreno, A., Perez, J., 2008, High iNOS expression in macrophages in canine leishmaniasis is associated with low intracellular parasite burden. *Veterinary Immunology and Immunopathology* 123, 353-359.
- Zerpa, O., Ulrich, M., Negron, E., Rodriguez, N., Centeno, M., Rodriguez, V., Barrios, R.M., Belizario, D., Reed, S., Convit, J., 2000, Canine visceral leishmaniasis on Margarita Island (Nueva Esparta, Venezuela). *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94, 484-487.

ABSTRACTS PRESENTED IN - IV INTERNATIONAL SYMPOSIUM ON DEVELOPMENTAL BIOLOGY AND III STUDENT MEETING ON DEVELOPMENTAL BIOLOGY



ORGANIZING COMMITTEE:

Dra Chao Yun Irene Yan - Universidade de São Paulo (ICB)
Dr. Cláudio Roberto Simon - Universidade Federal do Triângulo Mineiro (UFTM)
Dra Helena M. Araújo - Universidade Federal do Rio de Janeiro (UFRJ)
Dra. Ida Chow (SDB-USA)
Dra. Nadia Monesi - Universidade de São Paulo (FCFRP)



ORGANIZING COMMITTEE:

Ana Durvalina Bomtorin (USP)
Ana Paula Saita (USP)
Felipe Monteleone Vieceli (USP)
Livia Moda (USP)
Lucas Anhezini de Araujo (USP)
Maiaro Cabral Rosa Machado (USP)
Marcio Ribeiro Fontenele (UFRJ)
Ricardo Moraes Borges (USP)
Nadia Monesi (USP)

Time	Session	Speaker	Title
November 16th			
13:30-15:00	EABD	EABD	Interactive Round Tables 1
15:00-15:30		COFEE BREAK	
15:30-17:00		EABD	Interactive Round Tables 2
17:00-18:30	Workshop	Ida Chow (SDB)	Workshop on Strategies and Resources for Research and Teaching Developmental Biology
18:30-19:00		Happy Hour-Mixer	
19:00-20:00		DINNER	
20:00-21:00	Invertebrate Development	Ethan Bier Univ. California San Francisco	Dorsal-ventral patterning in the <i>Drosophila</i> embryo: from gradients to sharp lines.
21:00-21:40		Maria Luiza Paço Universidade de São Paulo	Ataxin-2 and A2bp1/Fox1 of <i>Drosophila</i> in stress granules and processing bodies
21:40-22:20		Claudio Simon Universidade Federal do Triângulo Mineiro	<i>jazigo (jaz)</i> : formins and cell death during development of <i>Drosophila</i>

November 17th			
7:00-8:00	Workshop	Ida Chow (SDB)	Strategies and Resources for Research and Teaching Developmental Biology
8:00-9:00	Invertebrate Development	Howard Lipshitz University of Toronto	Posttranscriptional regulation during early <i>Drosophila</i> development
9:00-9:40		Zilá P. Simões Universidade de São Paulo	Differential hind leg development in <i>Apis mellifera</i> castes
9:40-10:00		COFFEE BREAK	
10:00-10:40	Plant Development	Adriana Silva Hemerly Universidade	A novel regulatory network integrating cell division controls with plant development signals.

		Federal do Rio de Janeiro	
10:40-13:00	Workshop	Siobhan Brady UCDavis	Genomics and Development
10:40-11:30	Techniques and Technology	AOTEC	Macro Confocal in Developmental Biology: Leica TCS LSI
11:30-12:20		Carla Galindo Invitrogen	Microscopia de fluorescência e confocal: Faça seus resultados brilharem por muito mais tempo
12:20-13:10		Ana Flávia Pires Prodimol	RNA de interferência
13:00-14:00		LUNCH	
14:00-15:00	Plant Development	Siobhan Brady University of California, Davis	A tissue-enriched transcriptional network in the Arabidopsis root.
15:00-15:40		Márcio Alves Ferreira Universidade Federal do Rio de Janeiro	Uncovering novel regulators of Arabidopsis Stamen Development
15:50-17:50	POSTER		
17:50-18:30	Plant Development	Maria Helena Goldman Universidade de São Paulo	A novel tobacco stigma/style gene, <i>SC11</i> , encoding a lysine-rich protein that controls cell division and differentiation
18:30-19:30	Organogenesis	Shannon Fisher University of Pennsylvania	Transcriptional regulation of skeletal development and lineage in the zebrafish
19:30-20:30	Organogenesis	DINNER	
20:30-21:10		Milena Furtado Universidade Federal do Rio de Janeiro	The role of left-right axis on cardiac morphogenesis.
21:10-22:10		Peter Lwigale Rice University	The role of Semaphorin3A/neuropilin-1 signaling during vertebrate eye development.

November 18th			
8:00-9:00		Young Investigators Session	To be selected from abstract submissions
9:00-9:40	Evo-Devo	Tiana Kohlsdorf Universidade de São Paulo	Evo-Devo and the study of development genes in non-conventional model systems.
9:40-10:00		COFFEE BREAK	
10:00-10:40		Rodrigo Fonseca Universidade Federal do Rio de Janeiro	Evolution of dorsal-ventral axis formation in insects
10:40-11:40		Richard Behringer University of Texas	Developmental mechanisms of mammalian development
11:40-12:00		CLOSING SESSION	

Sponsored by



NOVEL REGULATORS OF THE HIF PATHWAY IN DROSOPHILA

Agustina Bertolin¹, Andres Dekanty and Nuria Romero

Fundación Instituto Leloir, Buenos Aires, Argentina

The hypoxia-inducible factors (HIFs) are a highly conserved family of transcription factors that constitute the major regulators of cellular oxygen homeostasis throughout the animal kingdom.

These factors target genes that are crucial for systemic hypoxia responses, such as angiogenesis and erythropoiesis, and cellular hypoxia responses involving metabolism, proliferation, motility and autophagy. HIF is a heterodimeric DNA-binding complex composed of a constitutively expressed HIF-beta subunit and a HIF-alpha subunit that is tightly regulated through multiple mechanisms. We have previously defined a hypoxia-responsive system in *Drosophila* which is homologous to HIF, being the proteins Sima and Tango the functional homologues of HIF-1 alpha and beta, respectively. Although the core HIF pathway and a few canonical regulators have been extensively characterized in the past decade, a systematic loss of function screen has not been performed so far.

Here we report an RNAi screen aimed to identify novel regulators of the hypoxic response. We used a *Drosophila* S2 cell line bearing a stably transfected luciferase reporter, which is strongly up-regulated upon exposure to hypoxia. We considered as potential regulators of the hypoxic response those genes whose interference led to a reduction of reporter activity, without affecting cell viability. The screen has allowed the identification of approximately 70 novel positive regulators of the HIF/Sima pathway. These included several members of the PI3K signalling pathway, genes of the Brahma chromatin-remodelling complex, and members of the AAA+ family of DNA helicases. Interestingly, we have also identified dATF-4 which is considered a central component in many cellular stress-response pathways. Finally, various members of the microRNA machinery scored as positives in the screen. We focused our studies on this last group of genes, and our results show for the first time that miRNAs may act as regulators of the hypoxic response.

MORPHOLOGICAL AND FUNCTIONAL STUDY OF CHOLESTEROL-ENRICHED MEMBRANE MICRODOMAINS DURING *XENOPUS LAEVIS* EARLY DEVELOPMENT

Alice Helena dos Reis², Leonardo Andrade, Karla Almeida, Mariana Louza, and Mirna Abreu e José Garcia Abreu

Programa de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, UFRJ, Rio de Janeiro, RJ

During vertebrate development, cell interactions are extremely important for correct

¹ abertolin@leloir.org.ar

² [alice](#)

formation of the anteroposterior and dorso-ventral axis. Although many different signaling mechanisms have been studied during early embryonic development, it is unclear how cells segregate these signals in order to achieve correct fate. In this respect cell membrane machinery play pivotal role in capturing and transducing cell signaling. Lipid rafts are membrane microdomains (MM) highly dynamic enriched for glycosylphosphatidylinositol-linked (GPI-linked) proteins, glycosphingolipids such as GM1 ganglioside and cholesterol. These structures are known for their ability to compartmentalize cellular processes and form large platforms for cell signaling. In order to study the MM function and organization during early crucial stages of embryonic development, Methyl-beta-cyclodextrin (M β CD) was injected into the blastocoele at blastula stage embryos to disrupt the MM. Phenotype analysis by Scanning electron microscopy showed that cholesterol depletion by M β CD caused anterior defects in *Xenopus* embryo: optic vesicles, cement gland, gills, oral cavity and forebrain were malformed. Despite this phenotype, transmission electron microscopy of the embryonic cells showed that the overall cell architecture was not affected, but some organelles such as lipid bodies and mitochondria were smaller. The *caveolin* and *flotillin* transcripts were identified during embryogenesis since early development until tadpole stages. Immunoassaying and dot blot analysis showed that lipid raft markers such as GM1 ganglioside, caveolin1 and flotilin1 were affected upon M β CD microinjection. Negative staining showed that lipid raft fraction obtained from M β CD injected embryos was not able to form organized and associated membranes, compared to those ones observed in non injected embryos. These results support that cholesterol-enriched membrane microdomains play crucial role during *Xenopus* anterior development and that cholesterol depletion by M β CD specifically affect the MM components and their ultrastructure, leading to head defects in *Xenopus* embryos.

Financial Support: CNPq, FAPERJ, CAPES, ICB-UFRJ.

DIFFERENTIAL DISTRIBUTION OF SOG FRAGMENTS REGULATES DPP SIGNALLING IN POLARIZED EPITHELIA OF DROSOPHILA MELANOGASTER

***Amanda Câmara³, Érika Negreiros, Marcio Fontenele
and Helena Araujo***

Institute for Biomedical Sciences, UFRJ, Rio de Janeiro, RJ - Brazil

How morphogen concentration gradients are established are fundamental issues in developmental biology of vertebrates and invertebrates. It is important because these gradients are responsible for determining territories from which cells differentiate into many tissues. Dpp (Decapentaplegic) protein is an extracellular morphogen and it has important roles during *Drosophila melanogaster* development. The generation of Dpp gradients depends on interactions with extracellular modulators such as the BMP-binding protein, Sog (Short gastrulation). Sog activity can be modulated by metalloproteases which cleaves it giving rise to different fragments: N-terminal and C-terminal. In addition, Integrin receptors can regulate the

³ amandacamara.ufrj@gmail.com .

Sog activity/mobility and consequently Dpp signaling in the pupal wing epithelium, impacting on the formation of wing veins.

In this study, we seek to understand how the distribution of Sog protein is regulated in polarized epithelia during follicular and pupal wing epithelia development, impacting on Dpp activity to pattern the dorsoventral axis of the embryo and vein formation, respectively. Immunostaining analysis show that Sog fragments have differential extracellular distribution during pupal wing development. Furthermore, subcellular distribution of Sog fragments is distinct at the apical and basolateral levels of wing cells. Subcellular Sog distribution in the follicular epithelium is also compartmentalized and it is dynamic according to different stages of follicular maturation. In addition, clonal analysis in follicular epithelium shows that the distribution of Sog fragments in the perivitelline space is regulated by Integrins, as it was previously reported in the wing epithelium. These results point to a conserved mechanism for the regulation of Sog distribution in the follicular and wing epithelia, based on the action of metalloproteases and Integrin receptors. This mechanism which controls the diffusible properties of Sog protein may be fundamental to spatially restrict Dpp morphogenetic activity in growing epithelia.

Financial Support: FAPERJ and CNPq.

ULTRABITHORAX EXPRESSION AND BRISTLE PATTERNING DURING POST-EMBRYONIC DEVELOPMENT OF HIND LEGS OF APIS MELLIFERA QUEENS AND WORKERS

Ana Duvalina Bomtorin⁴1, Angel Roberto Barchuk², Alexandre dos SantosCristino³, Livia Moda¹, Michelle Prioli Miranda Soares⁴, Marcela Ap. F. B.Laure¹, Rodrigo A. Faccioli⁵, Márcia Maria G. Bitondi⁴, and Zilá L. P. Simões⁴

1Depto. Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo

2Depto. Ciências Biomédicas, Unifal-MG

3IFSC, Instituto de Física de São Carlos, USP

4Depto. Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP

5EESC, Escola de Engenharia de São Carlos, USP

The queen-worker diphenism in the honey bee *Apis mellifera* results from differential feeding of female larvae. Adult workers have a structure on their hind legs that is used for carrying pollen and propolis, the corbicula (a central region of the tibia that is free of bristles), while queens lack this structure. Using scanning electronic microscopy we found that these leg structures are already formed in brown-eyed pupae in both castes. Oligonucleotide microarray analyses performed on prepupal hind legs showed that 68 genes are overexpressed in workers and 103 in queens, including genes related to juvenile hormone metabolism and the insulin

⁴ ana_durva@rge.fmrp.usp.br.

pathway. Using qRT-PCR we assessed the transcription pattern of some of these differentially expressed genes, as well as of others with already known participation in leg development: abdominal-A, ataxin-2, cryptocephal, dachshund, distal-less, grunge, Retinoic and fat acid Binding Protein (RfaBP) and ultrabithorax. ultrabithorax and abdominal-A had higher expression levels in prepupae and early pupae of workers than in queens. RfaBP expression was higher in queen than in worker prepupae. Using immunohistochemistry, we localized Ultrabithorax protein in the prepupal stage in the tibia and basitarsus of worker hind legs, as well as in the basitarsus of queens during prepupal and white-eyed pupal stages. We found that groups of cells in the basitarsus of white eyed queens pupae and workers prepupae were anti-Ultrabithorax negative. These cells, evidenced by DAPI, were arranged in a similar pattern to that observed in adult leg bristles, suggesting that the transient absence of Ultrabithorax is crucial in bristle morphology and patterning of the hind legs of *A. mellifera* females. We conclude that, based on their differential expression, abdominal-A and RfaBP are involved in leg structure development during caste differentiation in the honeybee, and we also evidence a specific role of ultrabithorax in this process.

Financial support Fapesp Proc. Nr. 2005/03926-5.

SUBCELLULAR LOCALIZATION OF JAZIGO IN DYING LARVAL SALIVARY GLANDS OF DROSOPHILA MELANOGASTER

***Ana Paula Saita⁵1, Lucas Anhezini Araujo1, Mara Silvia Alexandre
Costa1, Ricardo Guelerman Pinheiro Ramos1,
and Claudio Roberto Simon2***

Faculdade de Medicina de Ribeirão Preto –
USP Laboratório de Genética Molecular do Desenvolvimento
2Universidade Federal do Triângulo Mineiro – UFTM
Departamento de Ciências Biológicas

In a previous “forward genetics” screen we have identified a mutation that presents a drastic defective phenotype: the persistency of larval salivary gland (LSG). In wild type animals, LSG are fated to die in an autophagy-like programmed cell death (PCD) process early into the pupal stage of *Drosophila*’s development. This mutation was named jazigo (jaz). We mapped the affected gene and using bioinformatics a great homology between JAZ encoded proteint and components of the family of proteins called formins was seen. Formins are proteins involved in the active remodeling of actin-tubulin cytoskeleton in many different biological processes such as the establishment of cell polarity and cytokinesis. Formin’s signature is the presence of FH domains, most formins exhibit both FH1 and FH2 domains. The FH1 domains are proline rich domains and can serve as binding sites for SH3- and WW-motif containing proteins, as well as profilin. The FH2 domain is the most conserved domain

⁵ anasaita@usp.br.

and it has been related to the dynamics of actin polymerization. Due to the mutant phenotype and biological significance of formins, we have initiated the functional characterization of JAZ by generating polyclonal anti-JAZ antibodies in order to address its sub-cellular localization in dying LSG.

Immunoprecipitation assays revealed that JAZ is cleaved in dying LSG into two polypeptides (80kDa and 60kDa) that together add up to the expected size of the JAZ protein (~140kDa). Using confocal microscopy we could see that JAZ is initially scattered throughout the cytoplasm of dying LSG cells and at later stages of the process it migrates to the nucleus localizing in between polytene chromosomes. In persistent SGs of jaz mutants, the -JAZ staining revealed a "mosaic" pattern in which, those cells arrested at later stages of PCD, we could still see nuclear labeling, even though less intense, while cells arrested at initial stages of cell death, if present, we could see labeling only in the cytoplasm. These results are consistent with previous findings in HeLa cells that show FHOD1, a formin sharing the highest similarity to JAZ. FHOD1 has been shown to be cleaved and migrate to the nucleus. However its function, as well as JAZ functions into the nucleus, is still uncertain. The most obvious hypothesis would be that formins might play a role in disassembling the nuclear structure in such type of cell death. We are currently studying JAZ functionally in order to address this question.

Supported by FAPESP, CNPq and FAPEMIG

ESTABLISHMENT OF A MODEL SYSTEM TO STUDY THE VASCULOGENIC POTENTIAL OF PROEPICARDIA IN ADULT TISSUES

Bruno de Oliveira⁶ e José Xavier-Neto

Laboratório de Genética e Cardiologia Molecular, Instituto do Coração – InCor/FMUSP, São Paulo, SP

The Proepicardium (PE) is a transient extracardiac structure giving rise to all components of the coronary vessels. To evaluate the vasculogenic potential of the PE in an adult site, we transplanted a neonatal heart into the subcutis of an adult ear. Later, two PE from eGFP-transgenic mice were transferred to the surface of this heart. In another group, we transferred the PE directly into the ear pinna.

To evaluate the incorporation of eGFP cells derived from the PE, and to investigate their possible differentiation, we performed immunofluorescence for eGFP in combination with other markers: Cardiac Troponin-1 (cTn-1), Smooth Muscle alpha Actin (SMαA), PECAM/CD31 and endothelial Nitric Oxide Synthase (eNOS/NOSIII).

In the group that received the heart and the PE transplantations, double immunofluorescence against eGFP and cTn-1 showed extensive incorporation of eGFP+ cells in the periphery of the grafted heart. Double labeling with anti-SMαA revealed that eGFP+ cells are found internal to the medial smooth muscle layer and into the lumen, suggesting the incorporation of PE cells in the endothelial layer and in blood island hemangioblasts.

⁶ brhc@usp.br.

The endothelial contribution of PE cells was confirmed in the two groups by double staining with eGFP and PECAM, which is an early marker of endothelial and blood differentiation. Double staining with eGFP and eNOS, a terminal endothelial marker, was present only in the group that received heart transplantation with PE. Double eGFP/PECAM immunofluorescence revealed expression in intraluminal cells, consistent with the blood differentiation that is observed in coronary vessels precursors before overt endothelial differentiation.

Therefore, PE cells, can differentiate and likely participate in the process of neovascularization when transplanted to adult sites. These findings demonstrate that the vasculogenic potential of the PE cells is conserved in an adult site and our model is adequate to study the mechanisms involved in the development and regeneration of vasculature.

Financial Support: Capes, CNPq, Fapesp.

INACTIVATION OF THREE SUBFAMILIES OF TNT1, A TOBACCO'S RETROTRANSPOSON

**Danielle Maluf Quintanilha⁷, Bruno Karolski, Breno Lourenzzo,
Erika Maria de Jesus e Marie-Anne Van Sluys**

Departamento de Botânica, Instituto de Biociências, USP, São Paulo, SP

Transposable elements (TEs) are genetic units which can move within the host genome. They are present in large numbers within the genome of many multicellular organisms, and can constitute up to 70% of the genome in some plants. Tnt1 is a retrotransposon superfamily found in high copy number in the genome of tobacco and related *Nicotiana* spp. It is composed of three subfamilies which differ in their regulatory sequences, the "U3" region. The main goal of this work is to evaluate the biological role of Tnt1 in two *Nicotiana* species: *N. tabacum* and *N. benthamiana*. Genetic constructs designed to inactivate the retroelement through RNA interference were inserted in the genome of two species of *Nicotiana* by *Agrobacterium* transformation. The phenotypes of the regenerants, as well as in the filial plants, were compared to the wild type. Two generations of transgenic plants showed leaves with dead cell spots, reduced (or even absent) roots and seeds that lost viability more quickly than those of the wild type. Currently, more specific constructs are being designed in order to inactivate each Tnt1 subfamily individually based on the U3 region. It is known that each Tnt1 subfamily is preferentially expressed in different plant tissues and it is therefore hoped that inactivation of each Tnt1 subfamily will identify specific regulatory networks in the *N. tabacum* and *N. benthamiana* genomes. In parallel, histological root sections are being made in order to find possible differences in cell division between the transgenics and the wild type. Preliminary results showing that silencing generates aberrant plant phenotypes indicates that Tnt1 retrotransposons may play an important role of the development of the two *Nicotiana* species.

Financial Support: Pró-Reitoria de Pesquisa da USP.

⁷ dani_quintanilha@hotmail.com .

DAPPER1: A PARTNER OF THE WNT SIGNALING PATHWAY GENERATES TWO ISOFORMS DURING VERTEBRATES DEVELOPMENT

***Débora R Sobreira⁸ 1, José Xavier-Neto 2, Susanne Dietrich 3, Vera Nisaka Solferine 4, Marcio Roberto Pie 5, Frank R. Schubert 6 and
Lúcia Elvira Alvares 1***

1 Dept. de Histologia e Embriologia, Instituto de Biologia, Unicamp, Campinas, SP

2 Laboratório de Genética e Cardiologia Molecular InCor, HC.FMUSP, São Paulo, SP

3 School of Biomedical and Health Sciences, King's College London, London, U.K

4 Dept. de Genética e Evolução, Instituto de Biologia, Unicamp, Campinas, SP

5 Dept. de Zoologia, Universidade Federal do Paraná, Curitiba, PR

6 Institute of Biomedical and Biomolecular Sciences, School of Biological Sciences,
University of Portsmouth, Portsmouth, U.K.

Most of the developmental processes depend on the action of a few main families of paracrine factors, such as the Wnt proteins, that coordinate the organogenesis of metazoan embryos and will lead to the formation of complex organisms. The study of the molecules that interact with these paracrine factors is of extreme importance in order to understand their means of action. Dapper1 (Dpr1) is an adaptor protein that modulates the Wnt signaling during cephalic morphogenesis and mesoderm specification, through the interaction with Disheveled, a key protein of the Wnt pathway. Considering that adaptor proteins often use the alternative splicing machinery to increase proteomic variability, in the present study we used bioinformatic tools to search for Dpr1 isoforms in the vertebrate genome using the UCSC genome browser and databases. Initially, we aligned 127 mRNA and EST sequences of the mouse Dpr1 transcript with the provided RefSeq. The analyses showed that 10 of the sequences present an additional 111 bp residue, resulting in two putative isoforms, one longer and one shorter, which were named α and β isoforms, respectively. The inclusion of this segment to the Dpr1 mRNA adds 37 aa to its predicted protein sequence without disrupting the open reading frame, and is located in a non-conserved region of exon 4, known to be necessary for the interaction with the transcriptional factor Tcf3. In the next step we analyzed the genome of the other vertebrates and noted the presence of the two putative acceptor splice sites in the exon 4 of human, chicken and *Xenopus* Dpr1. In zebrafish, however, only a single acceptor splice site was identified. The existence of these two isoforms were confirmed by RT-PCR assays, suggesting that the balance between them may play an important role in the development of these organisms

Financial Support: FAPESP, CNPq and FAEPEX

⁸ deborarsobreira@gmail.com.

GENE ACTIVITY IN SALIVARY GLAND OF BRADYSIA HYGIDA (DIPTERA, SCIARIDAE): THE SECOND GROUP OF AMPLIFIED GENES

Diego Alves de Paula e Jorge Cury de Almeida⁹

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina, USP, Ribeirão Preto, SP

In the salivary gland polytene chromosomes of Diptera larvae, the RNA puffs are sites of intense transcription. Besides those puffs, the Scaridae also exhibit the DNA puffs, which are sites where gene amplification and transcription, developmentally controlled, occur. The hormone 20-OH ecdysone (20E) is involved in these controls. In *B.hygida*, two groups of amplified genes are present in two distinct groups of DNA puffs. The transcriptional activity of the first group demands high levels of 20E, while the second group needs low levels. Some genes of the first group are already cloned, but any of the second group. The DNA puffs A14, B3d, C6 and X4 (second group) start to expand and remain transcriptionally active in larvae from the age E7+12 hours to E7+24h, near to the pupal molt. Here, we are trying to produce and clone cDNA fragments of some genes of the second group. Poly(+)RNA was isolated from salivary glands of larvae at age E7+20h, when the second group of DNA puffs are active. The double strand cDNA was fractionated by electrophoresis in 1% agarose gel. The fractions were treated with Taq DNA polymerase and cloned in the vector pGEM-T Easy. PCRs, using the vector primers, were run with some colonies. From one of the samples, a small fragment was produced. This fragment was separated by electrophoresis, Southern blot was prepared. The fragment hybridized with a BhSGAMP-1 probe labeled with AlkPhos (GE). As the new fragment is longer than the original, it is possible that an isoform was cloned. BhSGAMP-1 encodes an antimicrobial peptide, which is expressed exclusively in the salivary glands and is developmentally regulated. We are waiting for the results of the fragment sequencing.

Financial Support: CNPq FAPESP FAEPA

TRIFLUOPERAZINE AND CHLORPROMAZINE, TWO PHENOTHIAZINES, REVERT MULTIDRUG RESISTANCE PHENOTYPE IN EMBRYONIC CELLS OF THE SEA URCHIN - ECHINOMETRA LUCUNTER – (LINNAEUS 1758)

Christiane Bezerra de Araujo, Elis Torrezan e Luís Fernando Marques-Santos¹⁰

Departamento de Biologia Molecular, Centro de Ciências Exatas e da natureza, UFPB, João Pessoa, PB

⁹ diegoaldpa@hotmail.com.

¹⁰ marques@dbm.ufpb.br

Phenothiazines are antipsychotic drugs used in the treatment of psychosis. These drugs exhibit a lot of pharmacological properties such as antagonism of dopamine D2 receptors, inhibition of calmodulin-dependent events and modulations of ABC superfamily proteins. ABC proteins are a large family of membrane proteins involved in the phenomenon of multidrug resistance (MDR). Previous works have shown a relevant activity of ABC proteins in embryonic cells of sea urchins. This study aimed to analyze the effect of two phenothiazines, trifluoperazine (TFP) and chlorpromazine (CPZ), in the resistance of embryonic development of the sea urchins *Echinometra lucunter* to the antimitotic drug vinblastine (VBL). Animals were collected at Cabo Branco beach (João Pessoa, PB - Brazil) and maintained in filtered sea water under constant air flow. Gametes were collected by intracoelomic injection of KCl (0.5 M). Embryos (1×10^4 embryos/mL) were cultured in 24-well plates at $26^\circ\text{C} + 2^\circ\text{C}$. After fertilization phenothiazines were added in concentrations ranging from 5 to 10 μM . Ten minutes later, VBL, a well-known ABC proteins substrate, was added at different concentrations (50 to 300 nM). At different time intervals, samples were collected and fixed in 4% paraformaldehyde. TFP and CPZ increased the sensitivity of embryos to VBL cytotoxic effect. MDR modulation was obtained with concentrations that did not block the early embryonic development. Furthermore, TFP was two times more efficient than CPZ. These results were similar to TFP and CPZ effects observed in MDR human tumor cell lines by other authors. Our work shows that TFP and CPZ were able of reverting MDR phenotype in the embryonic cell of *E. lucunter*, underlining the importance of ABC proteins in the maintenance of a biochemical barrier in sea urchin embryos.

Financial Support: CNPq

SILENCING OF TNT1 RETROTRANSPOSON RESULTS IN THE MODIFICATION OF THE EXPRESSION OF A WRKY53-LIKE GENE IN TOBACCO PLANTS

Erika Maria de Jesus¹¹, Bruno Karolski, Danielle Maluf Quintanilha and Marie-Anne Van Sluys

Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo

Tnt1 retrotransposons are present in solanaceae genomes in hundreds of copies. It becomes active when the plant is under specific stress conditions, such as treatment with methyl jasmonate or onozuka cellulase, and can be mutagenic. High copy number and activation under specific conditions suggests that Tnt1 may potentially be a key-element in the formation of gene regulatory networks. Its activation may impact the expression of cellular genes associated with host genome responses to specific stimulus. The silencing of Tnt1 through RNA interference resulted in tobacco plants with necrotic wound spots on leaves and delay in the development of roots. Based on the hypothesis that Tnt1 may be involved in pathways related to defense or stress response, as well as developmental processes, we

¹¹ erika_jesus@yahoo.com.br.

analyzed the expression of some genes involved in these processes, comparing tobacco wild type, Tnt1 antisense and Tnt1 silenced plants. Here we report the differential expression of a WRKY53 transcription factor-like gene in Tnt1 antisense and silenced plants. These results suggest a biological role for Tnt1, putatively involved in a network that includes the regulation of WRKY53. In *Arabidopsis thaliana* the WRKY53 transcription factor plays a central regulatory role in an early stage of leaf senescence, the final step of leaf development. Further large scale expression assays are necessary to identify other genes correlated with Tnt1 expression, in order to elucidate the mechanisms through this retrotransposon may impact the tobacco genome and developmental processes.

CRUSTACEAN EMBRYOS AS MODELS FOR RESPONSES TO ULTRAVIOLET-B RAYS

***Evelise Maria Nazari*¹²¹, *Dib Ammar*², *Valquíria Machado Cardoso*³, *Yara Maria Rauh Muller*³ e *Silvana Allodi*¹**

¹ Programa de Pós-graduação em Ciências Morfológicas, Instituto de Ciências Biomédicas, UFRJ, Rio de Janeiro, RJ

² Universidade do Oeste de Santa Catarina, UNOESC, Joaçaba, SC

³ Programa de Pós-graduação em Biologia Celular e do Desenvolvimento, Centro de Ciências Biológicas, UFSC, Florianópolis, SC

Exposure to ultraviolet-B radiation (UV-B) leads to increased cell damages in embryos that develop in shallow aquatic systems. Thus, we investigated responses of embryonic cells to UV-B rays using freshwater prawn embryos as models. Embryos at germinal disk stage (E3) were irradiated for 30min with 6W UVB lamp (dose 310 mW.cm-2). After, embryos were kept in the dark for 4 days, when they were analyzed at the initial morphogenesis stage (E7), which corresponds to embryos that still did not develop the eyes. Non-irradiated embryos at E7 were used as controls. The responses studied were: mitosis activity, apoptosis occurrence and the synthesis of the heat shock protein (hsp70), which may be produced as response to different environmental stressors. Immunohistochemistry with anti-phospho histone H3 revealed that the mitotic index (MI) shown by UV-B irradiated embryos (MI = 0.04, $P < 0.0001$) differed significantly from the controls (MI = 0.17). In addition, we observed that the embryonic structures visibly affected were the optic lobes and the naupliar appendages, i.e., the anterior part of the embryo. The TUNEL method showed that UV-B irradiated embryos presented an increased number of apoptotic cells also in the optic lobes and naupliar appendages. Immunohistochemistry using antibody anti-active caspase 3 demonstrated higher expression of this protein, specifically which be in the prospective nervous system cells at the anterior region of some UV-B irradiated embryos. Complementary immunohistochemical reactions revealed that UV-B irradiated embryos which did not express anti-caspase 3 showed an intense reaction with inducible anti-hsp70 in the prospective nervous system, which were identified with the Bp102 anti-axons antibody. Our results suggested that the UV-B rays changed the mitotic

¹² enazari@terra.com.br.

activity and induced apoptosis in the embryonic cells. Additionally, the UV-B effects were more evident in the nervous system cells, probably due to the required early differentiation of this system during the embryonic development.

CHICKEN SCRATCH2 IS EXPRESSED DURING EMBRYONIC NEUROGENESIS

***Felipe Monteleone Vieceli¹³, José Antonio Turri e Chao
Yun Irene Yan***

Dept. de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas,
Universidade de São Paulo, São Paulo, SP

In invertebrates, the Scratch (Srt) genes encode transcription factors that promote neurogenesis during development. The Srt function in vertebrates is unknown, but in mice Srt1 and Srt2 are specifically expressed in post-mitotic neurons in the embryo and in the adult central nervous system. In this work, we have cloned the coding sequence of chicken Srt2 (cSrt2) and characterized its expression pattern in the embryo with quantitative PCR and in situ hybridization.

The complete coding sequence was cloned in the expression vector pMES-GFP and the predicted translation product is a 276-aminoacids protein. The aminoacid sequence shares identities of 70% with rat Srt2 and 58% with zebrafish Srt. cSrt2 transcripts are firstly detected in the periphery of the neural tube in the hindbrain by HH 15 and in the spinal cord by HH 17, coinciding with the places where some of the first neurons differentiate during embryogenesis. Between HH 19-23, the expression in the motor domain of the spinal cord is progressively concentrated in the interface between the ventricular and mantle zones. Furthermore, cSrt2 expression is also observed in the dorsal root ganglia after HH22-23, particularly in the dorsomedial domain. The expression pattern of cSrt2 in the neural tube is complementary to that of Notch1, which is expressed in neural stem cells, and SCG10, a marker for differentiated neurons. Our results suggest that during embryogenesis cSrt2 is specifically expressed in post-mitotic undifferentiated neurons.

Financial Support: FAPESP

VITELLOGENIN KNOCKDOWN ENHANCES CASTE-SPECIFIC TRANSCRIPTIONAL DIFFERENCES IN HONEYBEE (APIS MELLIFERA) FAT BODIES

¹³ fmvieceli@usp.br.

**Francis M.F. Nunes^{1,4}, Angel R. Barchuk², Ana D. Bomtorin³,
Alexandre S. Cristino⁴, Márcia M.G. Bitondi¹, and Zilá L.P. Simões¹**

¹Departamento de Biologia - Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto – USP, Brazil

²Departamento de Ciências Biomédicas – UNIFAL, Brazil

³Departamento de Genética - Faculdade de Medicina de Ribeirão Preto – USP, Brazil

⁴The Queensland Brain Institute, Australia

Honeybees (*Apis mellifera*) exhibit remarkable caste-specific morphophysiological characteristics. Workers are short-lived, functionally sterile, performing all non-reproductive tasks in the colony. Queens are engaged in laying eggs and are long-lived. This dimorphism represents the phenotypic manifestation of the differential gene expression and developmental programs originated from the same genome. Thus, the proteome status appears to be a major determinant of honeybee reproduction and longevity, and the *vitellogenin* gene seems to play a crucial role. We used microarray approach to compare global gene expression differences between queen and worker abdominal fat bodies, where Vitellogenin is preferentially synthesized. The same comparison was conducted using orally-treated females with 500ng of double-stranded RNA for *vitellogenin* (dsVg) mixed with larval food during larval phase. Larvae were maintained in the colony until adult emergence. Queens were collected at emergence. Newly-emerged workers were maintained in an incubator at 34°C and a relative humidity of 80%, and were provided with water, pollen and sucrose syrup for seven days. Total RNA from abdominal fat bodies was extracted and used to synthesize aRNA probes for microarray slides hybridization. Results from ‘RNAi-treated’ group were subtracted from ‘untreated’ one. Changes in gene expression greater than 5 fold-change are reported. Sixteen genes were found to be overexpressed in dsVg-workers. From them, the main finding was the over-expression of two senescence markers, the $\alpha 1$ - and $\alpha 2$ -glucosidase genes. These data confirm our previous observation that *vitellogenin* disruption anticipates a specific age status, the foraging behavior. In queens, 27 genes were up-regulated, including three from *odorant binding* gene family, one *methyltransferase*, and the *vitellogenin* gene itself. In fact, we previously observed that *vitellogenin* silencing is less effective in dsVg-queens (~70% disruption) than in dsVg-workers (~90% disruption). A noteworthy observation was the reactivation of *hexamerin 70c* expression in dsVg-queen fat bodies. Usually, the expression of this storage protein gene ceases during larval phase. Taken together, our data show that the effects of the *vitellogenin* knockdown are more pronounced in dsVg-queens, thus contributing to the understanding of the molecular differences that distinguish honeybee castes.

Financial support: FAPESP 2005/03926-5 (ZLPS, MMGB) and 2007/07594-2 (FMFN); CNPq 473748/2008-8 (ARB) and Unifal-MG (ARB).

¹⁴ francis@rge.fmrp.usp.br

DESCRIBING A MECHANISM FOR THE INTERPRETATION OF THE POSITIONAL INFORMATION ESTABLISHED BY THE MORPHOGENETIC PROTEIN BICOID IN *DROSOPHILA* DEVELOPMENT

Francisco J.P. Lopes¹⁵,^{1,2}, Fernando M.C. Vieira,^{2,3} David M. Holloway,⁴, 5 Alexander V. Spirov¹, Paulo M. Bisch,²

1. Stony Brook University, United States of America.

2. Universidade Federal do Rio de Janeiro, Brazil

3. Universidade de Brasilia, Brazil.

4. British Columbia Institute of Technology, Canada.

5. University of Victoria, Canada.

During *Drosophila* embryonic development the concentration gradients of maternal factors, like the Bicoid protein, establish positional information along the embryo. A cascade of developmental genes reads out this positional information by exhibiting different activation levels according to their position along these gradients. One of the first genes of this cascade, the gap gene *hunchback* (*hb*) has a strong anterior expression and a sharp on-off boundary in the mid-embryo. It has been shown that Bcd binds to *hb* promoter cooperatively and that *hb* activates its own regulation. The role of Bcd cooperative binding for Hb pattern positioning has been demonstrated already, but the mechanism that allows the shallow Bicoid gradient to regulate the sharp Hb border remains a unsolved problem. We used a predictive kinetic model for *hb* transcriptional regulation, describing ligand binding/unbinding; combined with a series of experimental data altering Bcd cooperative binding and *hb* self-regulation ability [1]. We found that bistability stemming from *hb* self-regulation produces the sharp Hb border; the loss of sharpness for the *hb*^{14F} self-regulating mutant and the shallow pattern of a Bcd-dependent lacZ artificial construct support this result. In addition to that, our results indicate that Bcd cooperative binding determines the position of Hb pattern by determining the position where bistability occurs; our Bcd cooperative mutant data supports this conclusion. The ability to produce sharp borders is a central step for the expression of developmental genes in *Drosophila* and other organisms, and we show that spatial bistability can play a central role in this process.

[1] Lopes et al. PLoS Comput Biol. 2008 Sep 26;4(9).

Financial support: CNPq/MCT (Brazil), FAPERJ (Brazil), NSF/NIGMS grant 1-R01-GM072022 (USA), NIH grant 2-ROI-RR07801 (USA).

BHSAMP-1, A REITERATED GENE DEVELOPMENTALLY REGULATED IN THE SALIVARY GLAND OF BRADYSIA HYGIDA (DIPTERA,

¹⁵ fjplopes@gmail.com.

SCIARIDAE), ENCODES AN ANTIMICROBIAL PEPTIDE AND IS UNDER THE DIRECT CONTROL OF 20-OH ECDYSONE

Gabriela Morilha Zanarotti¹⁶ e Jorge Cury de Almeida

Departamento de Biologia Celular e Molecular Bioagentes Patogênicos,
FMRP, USP, Brasil

Animals and plants present as a first barrier, to resist infection by microorganisms, a sophisticated system of innate defenses. In insects, these systems include the production of large amounts of antimicrobial peptides (AMPs). Plants have also developed a wide variety of defense systems for protection against pathogens.

Recently we have shown that BhSGAMP-1 is a developmentally-regulated reiterated gene that encodes an antimicrobial peptide and is expressed exclusively in the salivary glands, at the end of the larval stage.

We are committed to characterize gene BhSGAMP-1 (for *Bradysia hygida* salivary gland antimicrobial peptide) and to investigate and demonstrate the role of 20-OH ecdysone (20E) in controlling BhSGAMP-1 activity.

In this study, we show, for the first time, that a gene for an antimicrobial peptide is directly activated by 20E. This control probably involves the participation of short-lived repressor(s). We also found that the promoter of BhSGAMP-1 is not equipped with elements that respond to infection, provoked by the injection of microorganisms, in the salivary glands or in the fat body. We produced polyclonal antibodies against the synthetic peptide and found that the BhSGAMP-1 peptide is secreted in the saliva. The BhSGAMP-1 gene was also activated during the third larval molt. These facts confirm our hypothesis that this preventive system of defense was selected to produce an environment free of harmful microorganisms in the insect's immediate vicinity, during molts.

Financial Support: CNPq, FAPESP, FAEPA.

IDENTIFICATION AND DISTRIBUTION OF HEPARANASE-1 IN THE RAT VENTRAL PROSTATE DURING THE FIRST WEEK OF POSTNATAL DEVELOPMENT

Guilherme Oliveira Barbosa¹⁷, Taize M Augusto, Alexandre Bruni-Cardoso, and Hernandes F Carvalho

Dept. of Anatomy, Cell Biology, Physiology and Biophysics - Institute of Biology - State University of Campinas, Campinas SP, Brazil

¹⁶ gabrielazanarotti@usp.br

¹⁷ gbarbosa.bio@gmail.com.

There is a sequence of events on ventral prostate (VP) development involving epithelial outgrowth, branching and canalization in parallel with epithelial cell differentiation and apoptosis (Anat Rec. 290:1223, 2007). During this process some enzymes acts on the extracellular matrix (ECM) remodeling. Matrix metalloproteinase-2 and -9 (MMP-2 and 9) are important in those events, either by degrading ECM or releasing cryptic components such as bioactive peptides and growth factors (Histochem Cell Biol 129:805, 2008). Heparanase (Hpse) is an endoglycosidase that degrades heparan sulfate, an important component of the basal membrane, which acts as a reservoir of growth factors regulating different aspects of cell physiology. We have hypothesized that heparanase-1 activity would have a relevant role in coordinating the dynamics of the epithelium and stromal remodeling during the early postnatal development of the rat VP. We have used In Situ Hybridization (ISH) at days 0 (date of birth), 3 and 6, and determined that heparanase-1 mRNA is concentrated in the epithelium, with no difference between proximal and distal part of the prostate ducts. The localization of the enzyme has been also demonstrated by immunohistochemistry. The enzyme is located at the surface of the epithelial cell and also adjacent to it including the basal membrane. Within these results we have knocked-down Hpse in organ culture, with siRNA, to analyze the effects of this gene silencing on total area and epithelium area of the VP. These findings show that this enzyme might have an important role during VP development, especially in those events related to epithelial growth and differentiation.

Financial Support: FAPESP

THE TRANSPOSON INSERTION IN DROSOPHILA LINE KG00562 INCREASES LETHALITY AND INTERFERES IN GENE EXPRESSION

Gustavo Borges Pereira¹⁸ and Maria Luisa Paço-Larson

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos
Faculdade de Medicina de Ribeirão Preto-USP Ribeirão Preto/SP

The transposon insertion site in *Drosophila* line KG00562 was mapped between loci CG8709 and *kermit*. Although studies of these evolutionarily conserved genes in different organisms indicate important roles in the control of lipid metabolism (CG879) and development (*kermit*), virtually no information exist on their functions in *Drosophila*. The CG8709, herein denominated DmLpin, encodes different isoforms. Previous studies have shown that a probe derived from the untranslated first exon of B isoform of DmLpin did not detected DmLpinB but rather a smaller 2.3Kb mRNA in the KG00562 mutant. It was also shown that the KG00562 mutant line presented high levels of lethality that were increased over the wild type background indicating a gain of function. Here we investigated the molecular bases of the mutation associated with the high lethality of the KG00562 line by sequencing the 2.3Kb mRNA and analysing the expression levels of DmLpinB and *kermit* in different genetic backgrounds. The sequence analyses, together with data on the expression profile of this

¹⁸ gustavo.borges10@gmail.com

transcript, have shown that the 2.3 kb mRNA is the result of an aberrant splicing between the first exon of DmLpinB and the second exon of the mini-white gene present in the KG construct. The expression of the chimeric mRNA seems not be deleterious since rescue of the normal lethality levels was observed in animals carrying KG00562 over a deficiency that covers the insertion region (Def7860). This result indicates instead that KG00562 mortality could be related with alterations in genes located in the vicinity of the insertion site. In agreement with this hypothesis qPCR analysis confirmed the absence of DmLpinB transcripts in all tissues analyzed and indicated overexpression of kermit in the intestinal tract (283%) and in Malpighian tubules (325%) of L3 larvae of line KG00562. The lack of DmLpinB expression does not contribute to the lethality of the KG00562 line since the animals carrying KG00562 over Def7860 present indices of mortality similar to the wild type. On the other hand, overexpression of kermit seems to be associated with the lethality of KG00562. Significantly higher levels of this gene transcript were detected in Malpighian tubules of animals carrying KG00562 over wild type background that present high mortality indices, but are reduced to levels similar to the wild type in the KG00562 over Def7860, which presents the mortality levels rescued to the normal levels.

INSIGHTS INTO THE ORGANIZATION OF DORSAL SPINAL CORD PATHWAYS FROM AN EVOLUTIONARILY-CONSERVED RALDH2 (ALDH1A2) INTRONIC ENHANCER

Hozana Andrade Castillo¹⁹, Roberta Mascioli Cravo, Ana Paula Azambuja, Marcos Sawada Simões-Costa, Sylvia Sura Trueba e José Xavier Neto

Departamento de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, USP, São Paulo, SP

Retinoic acid (RA) signaling is crucial for correct embryonic development and Raldh2 is the major enzyme involved in RA synthesis during early development. Using bioinformatics, mice transgenesis, chicken electroporation and *Xenopus* injection we detected an intronic enhancer that activates *raldh2* expression in spinal cord's roof plate and dorsal interneuron. Via site-directed mutagenesis we demonstrated that enhancer activities are established through a combination of dorsal stimulation via predicted double Tcf/vertebrate caudal-type, Cdx, homeobox binding sites, plus inhibition via predicted TGIF homeobox and ventral Lim-homeodomain repressor sites. Comparative studies this enhancer prompted description of a novel RA signaling domain in the dorsal spinal cord (SC). Double in situ hybridization with *raldh2* and *math-1*, or *cath-1*, indicates the existence of a transient and novel endogenous domain of *raldh2* expression in dII interneurons, which give rise to ascending circuits and intraspinal commissural interneurons. This suggests roles for RA signaling in the ontogeny of spinocerebellar and intraspinal proprioceptive circuits. To

¹⁹ hozanaac@gmail.com.

establish the phylogenetic representation of this novel RA signaling domain in vertebrates we performed *raldh2* in situ hybridization in agnathans and teleosts. We found that *raldh2* is expressed in dorsal interneurons throughout the agnathan SC, consistent with the transient expression of the gene in dorsal interneurons of tetrapods. This suggests an ancestral role of RA signaling in the ontogenesis of vertebrate intraspinal proprioception. Conversely, lack of *raldh2* expression in the SC roof plate of agnathans, which do not possess paired fins, and of *raldh2* expression throughout the SC in teleosts, which underwent reduction of their paired fins, suggests that roof plate RA signaling went missing with secondary simplifications, or loss of vertebrate paired fins/limbs.

Financial Support: FAPESP

CALCIUM INFFLUX IS REQUIRED FOR EARLY EMBRYONIC DEVELOPMENT OF THE SEA URCHIN ECHINOMETRA LUCUNTER

Jocelmo Cássio de Araujo Leite²⁰, Luis Fernando Marques-Santos

Departamento de Biologia Molecular, Centro de Ciências Exatas e da Natureza, UFPB, João Pessoa, PB

A transitory rise in cytosolic Ca^{2+} concentration in sea urchin eggs begins at sperm-egg fusion site and travels through the egg as a wave. Elevation of intracellular Ca^{2+} concentration is a vital signal for resumption of the cell cycle. It was shown that in most species studied, calcium influx is not essential for the initiation of Ca^{2+} wave and, therefore, it is not critical for embryonic cell division. In the present work, we evaluated the need of extracellular calcium in early embryonic development of the sea urchin *Echinometra lucunter*. Animals were collected at Cabo Branco beach, João Pessoa, northeast of Brazil. Eggs and spermatozoa were extracted with intracoelomic injection of KCl (0,5 M). Eggs were washed two times before fertilization to remove the jelly coat. Fertilization was induced by the addition of activated sperm suspension to eggs suspension. Two calcium chelators, EDTA and EGTA, were added to 1×10^4 embryos/mL five minutes after fertilization at different concentrations. The progression to the first and second cleavage was monitored under optical microscopy and 100 embryos were analyzed to each treatment. Both chelators inhibited development at all stages monitored. However, EGTA was more effective than EDTA in blocking the development. EGTA is a specific Ca^{2+} ion chelator, while EDTA is chelator for divalent cations. Considering that these chelators were able to block the early embryonic development, our data suggest that calcium influx is required for early embryogenesis of *E. lucunter*.

Financial Support: CNPq

²⁰ jocelmocassio@hotmail.com.

EFFECT OF THE CALCIUM IONOPHORE IONOMYCIN IN THE SEA URCHIN *ECHINOMETRA LUCUNTER* EGGS ACTIVATION

Jocelmo Cássio de Araujo Leite²¹, Elis Torrezan, Luis Fernando Marques-Santos

Departamento de Biologia Molecular, Centro de Ciências Exatas e da Natureza, UFPB, João Pessoa, PB

An increase in cytosolic calcium ion concentration at fertilization plays a key role in egg's activation. It was shown that in the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* fertilization membrane elevation was induced by treatment with calcium ionophore A23187. This fertilization-like change did not require calcium in the surrounding sea water, demonstrating that ionophore was releasing calcium from internal stores. In the present work we investigated the effect of the calcium ionophore ionomycin in *Echinometra lucunter* eggs activation in the presence or absence of external calcium ion. Sea urchins were collected at Cabo Branco beach, João Pessoa, northeast of Brazil. Eggs were collected with intracoelomic injection of 0.5 M KCl. Eggs were washed twice –ASW (with calcium) or ASW Ca^{2+} free (without calcium) - before activation to remove the jelly coat. Ionomycin was added to 1×10^4 embryos/mL at different concentrations. Eggs were monitored in optical microscopy at 1 and 5 minutes after ionophore addition. Ionomycin induced fertilization membrane elevation in ASW and ASW Ca^{2+} free. The elevation of fertilization membrane in ASW was evident since from 1 μM . Five times more ionomycin was needed to induce the same response when eggs were cultured in ASW Ca^{2+} free. These data show that ionomycin was able to induce calcium ions mobilization from internal stores and from the surrounding sea water. Our data also suggest that the calcium concentration present in intracellular stores is sufficient to promote egg activation in *Echinometra lucunter*

INDUCING OF A “ZONE OF POLARIZING ACTIVITY (ZPA)-LIKE” IN THE FIRST BRANCHIAL ARCH BY SONIC HEDGEHOG

**José Marques de Brito²²¹, Marie-Aimée Teillet²
and Nicole Le Douarin³**

1Laboratório de Embriologia de Vertebrados, Instituto de Ciências Biomédicas, CCS, UFRJ, Rio de Janeiro, RJ

2 Laboratoire de Développement, Evolution et Plasticité du Système nerveux, Institut de Neurobiologie Alfred Fessard, UPR 2197, CNRS, F-91198 Gif-sur-Yvette, France

3 Laboratoire de Biologie du Développement, UMR 7622, CNRS, Paris, France

²¹ jocelmocassio@hotmail.com

²² brito@histo.uftj.br.

Recently we have shown in chick embryos that Sonic hedgehog (Shh) derived from ventral foregut endoderm plays a critical role in the development of the first branchial arch (BA1) derivatives by two distinct ways: first by ensuring the survival of mesenchymal cells derived from the neural crest (NC), second, by patterning the oral ectoderm.

To further explore the role of Shh on BA1 development, we performed grafts of quail fibroblasts transfected with a *Shh* construct (QT6-Shh cells) into the presumptive BA1 territory in 5-6 somites stages (ss) chick embryos. At E3-4 the experimental side was hyperplastic and showed an expanded domain of *Fgf8* and *Bmp4* expression in BA1 ectoderm and an ectopic expression of Shh. This effect was also observed in the mesenchyme in which the transcription of *dHand*, *Dlx5*, *Sox9* and *Pitx1* was increased. Later on, at E11 supernumerary lower jaws developed in the side where QT6-Shh cells were grafted with a mirror image polarity. Moreover, the growth of supernumerary skeletal elements derived from BA1 (eg. Meckel's cartilage) was preceded by the ectopic expression of *Bmp4* in the distal part of BA1 ectoderm.

Altogether our results confirm that Shh is important in patterning of BA1 ectomesenchyme. We have evidence that the effect of *Bmp4* on NC cells consists in insuring their survival. On the other hand Shh seems responsible for the induction of *Bmp4* expression in BA1 ectoderm. *Bmp4* itself controls the growth of supernumerary skeletal pieces.

Financial support: FAPERJ, CNRs and ARC.

IDENTIFICATION OF RING GLAND TRANSCRIPTIONAL ACTIVATORS

Juliana Candido-Silva²³ and Nadia Monesi

1Departamento de Análises Clínicas, Toxicológicas e Bromatológicas,
Faculdade de Ciências Farmacêuticas de Ribeirão Preto,
Universidade de São Paulo, São Paulo, Brasil

The BhC4-1 gene is expressed in *Bradysia hygida* salivary glands at the end of fourth larval instar. The mechanisms that control the BhC4-1 salivary gland expression are conserved in transgenic *Drosophila*. The functional characterization of the DNA puff BhC4-1 promoter revealed that the proximal promoter contains both a 129 bp (-186/-58) salivary gland enhancer and a 67 bp (-253/-187) ring gland enhancer that drive tissue specific patterns of developmentally regulated gene expression. In transgenic *Drosophila*, BhC4-1-lacZ expression in the ring gland is initially detected in late embryos, continues during the larval stage and prepupal stage, and is no longer detected in 24h pupae. The BhC4-1 ring gland enhancer is, to our knowledge, the only identified enhancer that is able to drive developmentally regulated gene expression exclusively in the ring gland of late embryos. The ring gland is the main larval endocrine organ and the mechanisms that control gene expression in this organ are largely unknown. In order to characterize transcriptional activators that act in the ring gland, we have employed the yeast one-hybrid system to identify proteins that are able to bind the 67pb BhC4-1 ring gland enhancer. The initial screen was performed in the presence of 40mM 3-AT, and

²³ julianacandido@aim.com.

257 positive clones were identified. In order to eliminate false-positive clones we used the lacZ gene as a second reporter gene which reduced the number of initial 257 positive clones to 190 clones. Ninety two percent of the positive clones that are able to activate both reporter genes in the yeast one-hybrid system were sequenced. Preliminary results reveal that 112 clones constitute unique sequences associated with annotated genes (CGs) of *Drosophila melanogaster*. The 112 sequences were classified according to the category Biological Process of Gene Ontology as follows: cell cycle and DNA replication (7.2%), cell transport (7.2%), cytoskeleton organization (10%), extracellular matrix and morphogenesis (4.5%), metabolic process (14%), other functions (4.5%), signal transduction (4.5%), transcription (8%), translation (24.1%) and unknown functions (16%). The functional validation of the sequences identified as transcriptional factors is currently being performed.

Financial support: FAPESP, CNPq, PRP-USP.

EFFECTS OF FOLIC ACID AND HOMOCYSTEINE ON CELL PROLIFERATION, APOPTOSIS AND NCAM EXPRESSION IN THE SPINAL CORD OF THE CHICKEN EMBRYO

Karoline Kobus²⁴¹, Evelise Maria Nazari², Neide Armiliato³ e Yara Maria Rauh Müller¹

¹ Programa de Pós-graduação em Biologia Celular
e do Desenvolvimento, Centro de Ciências Biológicas,
UFSC, Florianópolis, SC

² Programa de Pós-graduação em Ciências Morfológicas,
Instituto de Ciências Biomédicas, UFRJ, Rio de Janeiro, RJ

³ Universidade do Contestado, UnC, Concórdia, SC

Neural tube defects (NTD) comprise disorders of primary neurulation and are among the most distressing congenital anomalies. These anomalies originate from failure of neural tube closure or from abnormalities of the axial mesenchyme development. Maternal ingestion of folic acid (FA) reduces NTD, which are associated with high homocysteine (Hcy) levels. This study evaluated the effects of FA and Hcy on cell proliferation, apoptosis and cell adhesion during development of the spinal cord and mesenchyme. Fertile eggs of *Gallus domesticus* were incubated (38°C) and after 30 h, embryos were divided into 3 groups (n = 10 per group) and submitted to treatments by the injection into the yolk sac: group I - 25 µl saline with 0.5 µg FA; group II - 25 µl saline with 10 µmol D,L-Hcy; group III - 25 µl saline with 0.5 µg FA and 10 µmol D,L-Hcy. Control embryos were injected with 25 µl saline. After 96 h of incubation, embryos were anesthetized at 4°C, removed from the egg membranes and fixed in formaldehyde. TUNEL staining was used to identify apoptotic cells and immunohistochemistry was performed to identify the proliferating cells (anti-phospho histone

²⁴ karolinekobus@gmail.com

H3) and NCAM (anti-NCAM) expression. Stereological analysis was performed using the M-42 test system (Weibel N° 2) to evaluate whether the treatments induced apoptosis or affected cell proliferation. FA-treated embryos showed the highest numerical density of apoptotic cells in spinal cord ($68.76 \pm 9.43 \text{ mm}^2$). FA reversed the decrease of proliferating cells in the spinal cord induced by Hcy ($45.84 \pm 4.71 \text{ mm}^2$). Hcy treatment reduced NCAM expression and FA prevents this effect. The results demonstrate in situ that the imbalance between FA and Hcy levels can lead to disruptions in spinal cord and mesenchyme development, changing proliferation, apoptosis, and cell adhesion and consequently changing the arrangement of the spinal cord layers.

Financial Support: CAPES

LIMBLESSNESS AND HOXA-13 EXPRESSION IN PYTHON MOLURUS

Lígia Tereza Bertolino²⁵ e Tiana Kohlsdorf

Dept de Biologia, Faculdade de Filosofia Ciências e Letras,
USP, Ribeirão Preto, SP

The origin of Snake clade in squamates coincided with some morphological changes, such as limb loss, body elongation and the appearance of the cloacal scent glands. The evolution of this body form in Squamates, was likely associated to modifications in parameters related to embryonic development, for example in the expression pattern of homeotic genes. In limbless organisms, the homeotic genes involved in limb development might be maintained in the genome due to pleiotropic effects on other traits that are of selective value to this organisms. One example would be Hoxa-13, a transcription factor involved in autopodium development. In snakes, a possible scenario would be that, after limbs were lost, the selective pressures were relaxed on specific sites of the sequence of Hoxa-13 associated to limb development. However, a recent study (Kohlsdorf et al, 2009) has detected a consistent signal in Hoxa-13 sequences associated with origin and loss of limbs in several species of Gnathostomata. This pattern of molecular evolution suggests that, even if selective pressures were relaxed immediately after limbs were lost in snakes, positive and directional selection began to influence the evolution of this gene at some point of the evolutionary history of the clade. Moreover, such evidence of directional selection in Hoxa-13 might suggest that the appearance of evolutionary innovations became possible after a specific selective pressure (related to autopodium development) was relaxed when limbs were lost. In this context, the expression of Hoxa-13 in snakes could be associated to the appearance of a new trait, for example, the cloacal scent glands. This study aims to analyze the Hoxa-13 expression in different developmental stages of Python molurus embryos, with a subsequently comparison to expression patterns in the limbed species Gallus gallus and Iguana iguana. Hoxa-13 expression is observed in specific regions of the embryo (pectoral girdle, anterior and posterior limbs in Gallus gallus e Iguana iguana case, post cloacal region, and restant region of tail) using

²⁵ ligia_bertolino@hotmail.com

specific primers in a Reverse transcriptase polymerase chain reaction (RT-PCR). This study represents a landmark in Evo-Devo for combining information from bioinformatics with morphological and molecular data in a comparative setup.

Financial Support: FAPESP

BRAIN STRUCTURES DEVELOPMENT OF *APIS MELLIFERA* WORKERS IS DELAYED COMPARED TO QUEENS

Livia M. R. Moda²⁶¹; Ana D. Bomtorin¹; Marcela A.B.F. Laure¹; Zilá L.P. Simões²

¹Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto, SP.

²Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP, Ribeirão Preto, SP.

Honeybee female larva has the capacity to develop into two different phenotypes: queens or workers. Differential nutrition triggers the development of caste-specific characteristics, such as body size and mushroom bodies/brain volume which are greater in workers than in queens. In adults, mushroom bodies are related to memory and learning, and are connected to tasks outside the colony led by workers. They are present in both sides of the protocerebrum and are composed of two pedunculi with a calyx in each one. These pedunculi and calyces are formed by dendrites and axons of Kenyon cells. Despite our understanding of adult brain structures, little is known about its development during early larval instars. To investigate whether differences could be detected in brain development in the context of caste differentiation, we performed high-resolution neuroanatomical studies of whole mount preparations of queens and workers larval brains. We used actin phalloidin/rhodamin to visualize axons and DAPI to visualize nuclei. In third instar larvae the most pronounced features are axons from optic tubercles, antennal lobes and inter tubercle tract, and also clusters of neuroblasts nuclei in each side of the brain. No morphological differences between queens and workers were detected at this stage. In fourth instar, phalloidin/rhodamin staining revealed the appearance of the pedunculus in the queen's brain whereas it is still absent in workers. In the fifth instar, pedunculus appears in worker brain and the formation of calyces of neuropils started in the queen's brain. Our results revealed that during larval development a delay is present in the formation of worker brain structures compared to queen. These caste-specific neurogenic events result from differential nutrition offered to larvae during early stages.

Financial support: FAPESP 2005/03926-5 (ZLPS) and 2009/00810-7 (LMRM)

²⁶ liviamoda@usp.br

TRANSCRIPTIONAL SILENCING OF JAZIGO USING IN VIVO RNA INTERFERENCE

**Lucas Anhezini de Araujo²⁷1, Ana Paula Saita¹, Ricardo G. P.
Ramos¹ and Claudio R. Simon²**

1- Departamento de Biologia Celular e Molecular e Bioagentes
Patogênicos – FMRP –USP

2- Departamento de Ciências Biológicas - Universidade Federal do
Triângulo Mineiro – UFTM

During metamorphosis of *Drosophila* two pulses of the steroid hormone 20-hydroxycdysone regulate stage and tissue-specific responses such as cell death and differentiation. While the first pulse triggers the larval midgut cell death and differentiation of CNS the second pulse is responsible for triggering larval salivary gland autophagic cell death. Trying to address how a systemic signal is responsible for the different cell responses during metamorphosis, we have assumed that several uncovered genes would participate in modulating such distinct responses. In order to answer that we performed a “forward genetics” screen looking for mutations affecting the salivary gland cell death process. Screening the collection of 1475 enhancer trap lines (P-element insertions) we found that one of these insertions (mutation) drastically affects the programmed cell death (PCD) of larval salivary glands during metamorphosis. The gene flanking the P-element insertion (affected gene) was named *jazigo* (*jaz*) and encodes a protein which shares high homology to FH proteins (“formin homology”) usually called formins. Our transcriptional analysis revealed that *jaz* is expressed in response to the second pulse of ecdysone in a similar fashion to other cell death genes. Formins had been related to the actin and tubulin cytoskeleton remodeling in different biological processes. Based on the phenotype, transcriptional pattern and *jaz* regulation by ecdysone we are interested in understanding how such protein functions in the cell death of larval salivary glands. To address that we have generated modular transgenic RNAi fly lines. The induction of transgene expression resulted in increased embryonic and larval lethality when compared with control animals. Furthermore, the dsRNAi expression during metamorphosis using controlled heat-shock produced salivary glands that failed to die (persistent salivary glands). The cytoskeleton analysis of these persistent salivary glands showed a distinct aspect of disassembling of the actin cytoskeleton when compared to other *jazigo* mutant alleles and dying wild-type salivary glands.

Financial Support: FAPESP, CNPQ e FAPEMIG

DPR1 AND DPR2 HAVE A DYNAMIC EXPRESSION PATTERN DURING LIMB DEVELOPMENT IN CHICKEN (GALLUS GALLUS)

²⁷ lucasanhezini@usp.br

Lúcia Elvira Alvares²⁸1, Denner Peterlini¹, Débora R. Sobreira¹, José Xavier-Neto², Susanne Dietrich³ and Paulo Pinto Joazeiro¹

1. Dept. Histologia e Embriologia, Instituto de Biologia, UNICAMP, Campinas, SP

2. Lab. Genética e Cardiologia Molecular, Inst. do Coração, Incor - HC, FMUSP, São Paulo, SP

3. School of Biomedical and Health Sciences, King's College London, London, U.K.

The Dapper proteins (Dpr) are multifunctional molecules coded by a family of genes that are present only in the genome of vertebrates. These proteins are required for key processes of development, such as morphogenetic movements, mesoderm and neural tissue specification, heart and eye morphogenesis. In chicken, two Dpr genes (Dpr1 and Dpr2) were identified and their expression patterns were established during early development. The limb bud was one of the structures of the chicken embryo identified as having the expression of both Dpr genes. This fact suggested that at later stages of development these molecules could also be expressed in the limbs. To explore this hypothesis, we performed whole-mount in situ hybridization of chicken embryos from HH21 to HH32.

The expression of Dpr1 and Dpr2 was clearly evident in limb buds from HH21 onwards. Between HH21 and HH25 transcripts were associated with the limb mesenchyme however avoiding the central core that forms the cartilage/bone elements. A strong downregulation of Dpr1 expression was identified at later stages of development where Dpr1 mRNAs were restricted to a narrow region around the growing bones. In comparison to Dpr1, Dpr2 displayed a more dynamic expression pattern. At HH25, Dpr2 transcripts were specifically identified in the cartilage rudiments that form the limb bones. At later stages, Dpr2 was expressed at the joints, at the tips of the digits and in tendons. Overall, these results revealed that Dpr1 and Dpr2 are expressed in a dynamic fashion during limb development in chicken, indicating that the activity of these genes may be important for the ontogeny of these structures. Further analyses are being conducted to identify the specific tissues expressing the Dpr genes in the limbs environment.

Financial Support: FAPESP

CONTRIBUTION OF THE PRECHORDAL PLATE MESODERM PRODUCING SHH TO THE ANTERIOR FOREGUT ENDODERM FORMATION

Luiz Felipe Lima da Silva²⁹, Alice Helena dos Reis and José Marques de Brito-Neto

Laboratório de Embriologia de Vertebrados, Instituto de Ciências Biomédicas, CCS,
UFRJ, Rio de Janeiro, RJ

²⁸ lealvare@unicamp.br

²⁹ silveiralf@hotmail.com.

The prechordal plate (PcP) is a transitory embryological structure formed by migrating cells derived from Hensen's node and localized between the neuroectoderm and the endoderm. Later on, PcP cells will interact with the endoderm and may be called "mesoendoderm". PcP is an organizing center for the forebrain development and is the first source of SHH in the most anterior region of the embryo. Using the chick embryo as a model, we have demonstrated that SHH produced by the anterior foregut endoderm is critical for lower jaw development. In the chick, *Shh* expression in the foregut endoderm can be detected at 4-5ss, in its most anterior part, in close contact with the prechordal plate (PcP). Furthermore, we showed that the forehead ablation including the anterior foregut endoderm and the PcP hampers *Shh* expression in the first branchial arch (BA1) endoderm and the lower jaw development.

Our previous works showed that, during encephalic neural crest cell migration, only the anterior ventral endoderm, which contacts PcP, expresses *Shh*. However, it's not known whether the anterior endoderm is capable of secreting *Shh* or it depends on the PcP induction. In this study, we analyze the contribution of the PcP for the anterior foregut endoderm. We describe the kinetics of *Shh* expression on histological sections from HH4 to HH9 as a way of detecting molecular limits of the prechordal plate. We applied the Schiff procedure to see if the localized expression of *Shh* was due to the presence of a basal lamina, which characterizes tissue distinctiveness. We also show scanning electron micrographs of the PcP in the search for morphological differences between the tissues composing it and the precise timing of the mesoendoderm formation. In this work, we did a descriptive approach to better understand the interaction between the PcP and the anterior foregut endoderm.

In the present work, we show preliminary results pointing that the PcP cells become integrated to the anterior endoderm at HH5. These data suggest that the PcP cells rather than the endoderm will be responsible for SHH production in the anterior ventral endoderm.

Financial support: CNPq e FAPERJ

A STUDY OF THE TRANSCRIPTIONAL REGULATION OF THE *ROUGHEST* GENE DURING THE DEVELOPMENT OF *DROSOPHILA MELANOGASTER* COMPOUND EYE AND THE MOLECULAR CHARACTERIZATION OF *ROUGHEST DOMINANT (Rst^D)* REVERSIONS

**Maiaro C. R. Machado³⁰; Shirlei Octacílio-Silva; Mara S. A. Costa;
Ricardo G. P. Ramos**

Biologia Celular e Molecular e Bioagentes Patogênicos, FMRP-USP

Previous phenotypic characterization of *rst^D*, a dominant regulatory *rst* allele, showed that the correct expression of Rst protein is essential for pigment cell specification during the final stages of compound eye development. *rst^D* also shows a high reversion rate, either to rough or wild type-like eyes. Here we report additional molecular and transcriptional characterization of this mutant and its revertants using Southern blotting and quantitative real time PCR.

³⁰ e-mail: maiaro@usp.br.

Although Southern Blot analyses detected high molecular weight bands in *rst^D* and nearly all of its revertants that are absent from wild type genomic DNA, no significant difference between wild type and wild type-like revertant *rst^{DRWT#6}* could be seen. *rst^{DRWT#6}* sequence analysis showed only single nucleotide alterations, besides a small 10-12 nucleotide deletion inside the genomic region where *rst^D* regulatory mutation was previously mapped. Additionally *rst* transcriptional dynamics of retinas from wild type, *rst^D* and *rst^{DRWT#6}* at 0, 25 and 35% pupal development (pd) were examined by Real Time PCR. The preliminary data obtained so far indicates that *rst* mRNA levels in *rst^D* mutants are slight higher than wild type at the time of cell sorting (25% pd) while in *rst^{DRWT#6}* revertants it is at basal levels in all three stages. These results lend support to earlier data from our laboratory indicating that the timing of expression, rather than the absolute amount of Rst, is critical for its function in the pupal retina.

Financial Support: CAPES and FAPESP

STUDY OF PUTATIVE MALE FERTILITY GENES LOCATED ON THE Y CHROMOSOME OF *D. MELANOGASTER* USING DOUBLE-STRANDED RNA INTERFERENCE TECHNOLOGY (RNAI)

Márcio Augusto Buffolo¹, Antônio Bernardo de Carvalho³¹², Helena Marcola Araujo³

^{1,3}Institute of Biomedical Sciences, Federal University of Rio de Janeiro - Brazil

²Department of Genetics, Federal University of Rio de Janeiro – Brazil

In *Drosophila*, sex not is determined by the Y chromosome. However, the Y is necessary for male fertility since males lacking a Y chromosome are viable but completely sterile (BRIDGES 1916). The *Drosophila* Y chromosome was the first heterochromatic chromosome to be characterized genetically and cytologically. 6 chromosomal regions have been characterized, each harboring at least one gene responsible for male fertility. (BROSSEAU, 1959). Because of its heterochromatic character, the discovery of new genes has been slow. Carvalho et al, 2001 and 2008 described 3 genes expressed exclusively in males that may be responsible for male fertility. They are coined ORY (Ocludin Related Y), CCY (Coiled-Coils Y) and WDY (WD-40 Y), that map to regions ks-1, ks-2 and kl-1 respectively. Classic studies of gene function involve the generation of loss-of-function alleles and the evaluation of mutant phenotypes. However, due to its heterochromatic nature, the generation of mutations in loci located in the Y is very complicated. For this reason, we have used the technique of interference by double-stranded (ds) RNA to knockdown the expression of the loci cited above. We have generated *Drosophila* strains bearing insertions of a pWIZ vector containing repeated and inverted sequences of the genes of interest under the control of a UAS promoter. For generation of the dsRNA, expression was directed by use of a GAL4 driver under the control of a testicular promoter (nos-GAL4VP16, expressed in the germline). Effects on male

³¹ bernardo@biologia.ufrj.br

fertility were evaluated by counting the number of viable embryos generated from crosses between wild type females and nosGal4>UAS-(gene of interest) males, and compared to appropriate controls (wild-type and nosGAL4). Our initial experiments indicate that all 3 genes tested reduce the number of viable embryos and thus may be involved in male fertility. These essays are being repeated with the same and additional GAL4 drivers. The expression pattern of the genes tested will be presented by in situ hybridization as well as alterations in the levels of expression of ORY, CCY and WDY by Real Time-PCR.

This work is supported by FIRCA/NIH, PRONEX and FAPERJ.

MATERNAL DPP/BMP PATHWAY REGULATES CALPAIN A ACTIVITY TO PATTERN THE EMBRYONIC DORSAL-VENTRAL AXIS IN DROSOPHILA MELANOGASTER

Marcio Fontenele³² and Helena Araujo

Institute for Biomedical Sciences – UFRJ, Rio de Janeiro, RJ – Brazil

Dorsoventral (DV) patterning in *Drosophila melanogaster* is regulated by the Toll pathway by modulating Cactus/I κ B protein degradation, and thus nuclear Dorsal/NF κ B protein levels in the early embryo. In addition to the Toll pathway, previous genetic studies have shown that the maternal Dpp/BMP pathway regulates nuclear Dorsal levels by controlling signal-independent degradation of Cactus. In this work, we show that the maternal Dpp pathway requires Calpain A, a calcium-activated cysteine protease, to target Cactus degradation. We find that Calpain A can regulate Dorsal levels by targeting Cactus protein. In addition, the Cactus PEST domain, which is needed for signal-independent degradation, is also required for Calpain A to exert an effect on Cactus. Using Real-time qPCR and biochemical approaches we show that the maternal Dpp pathway regulates the activity and/or mRNA stability/translation of Calpain A in early embryos. These results suggest that maternal Dpp functions through a non-canonical pathway, since the effects of Dpp blockage are observed before the onset of zygotic transcription. We also search to define the functional role of maternal Dpp on DV patterning. in situ hybridization and immunofluorescence analysis in embryos with disrupted Dpp pathway suggests that Dpp signals regulate discrete nuclear Dorsal levels. This raises the possibility that Dpp provides robustness to DV patterning.

Financial Support: CNPq and FAPERJ.

LIPIDOMIC ANALYSIS IN *XENOPUS LAEVIS* DEVELOPMENT

³² marciofontenele@yahoo.com.br or haraujo@histo.ufrj.br.

Mariana Pinto Louza³³¹; Alice Helena dos Reis¹; Karla Loureiro Almeida Coburn¹; Dangelo Carlo Magliano¹; Mirna Soares Abreu¹; Marcelo Einicker-Lamas²; Geórgia Atella³ e José Garcia Abreu¹

¹Instituto de Ciências Biomédicas-UFRJ

²Instituto de Biofísica Carlos Chagas Filho-UFRJ

³Instituto de Bioquímica Médica-UFRJ

The cell membrane has an important role in cell signaling, and there are specific regions that act as platforms for aggregating signaling molecules, the cholesterol-rich membrane microdomains (MM) or lipid rafts. Our group has recently shown that cholesterol depletion by Methyl-beta-cyclodextrin (M β CD) affects anterior development of *Xenopus* embryos, suggesting a correlation between MM and signaling pathway necessary for head formation. This work aimed to analyze lipid composition during the development of *Xenopus* embryos, and specifically how they appear in the MM.

Ventral and dorsal halves of *Xenopus* embryos at blastula and gastrula stages were analyzed by Thin Layer Chromatography (TLC). We observed a major lipid concentration at the ventral side, at the gastrula stage, but at blastula this difference was not so evident. In order to isolate MM fractions, embryos at four cells, blastula, gastrula and early neurula stages were lysated and submitted to a sucrose density gradient. Cholesterol measurement analysis demonstrated that this fraction has a peak of this lipid at blastula and gastrula stages, suggesting that MM organization might occur in a specific time point. TLC revealed that MM are mostly composed of cholesterol esters, triacylglycerols, phosphatidylethanolamines and phosphatidylcholines. Stage 9 *Xenopus* embryos (blastula) were microinjected or not with 40 mM M β CD and allowed to develop until gastrula stage, and the embryo extracts were then passed through a sucrose density gradient. Analysis of protein concentration and SDS-page revealed no drastic differences between MM fractions obtained from injected and uninjected control embryos. However, analysis of neutral lipids by TLC showed that M β CD microinjection increased the fatty-acids content, while decreased the triacylglycerol.

This work analyzed for the first time the lipid content during early *Xenopus* development. Our results suggest that there is a qualitative distribution of lipids along dorsal and ventral sides of the embryo. We also investigated the lipid content of the *Xenopus* MM during embryonic development and observed that cholesterol depletion affected neutral lipid composition. Since we observed a high content of phospholipids in MM, we also suggest that these structures may be involved in cell signaling during *Xenopus laevis* development.

Financial Support: FAPERJ, CAPES, CNPQ

SUBCELLULAR LOCALIZATION OF DFOX-1 IN THE VISUAL SYSTEM OF DROSOPHILA MELANOGASTER DURING DEVELOPMENT

³³ marianalouza@anato.ufrj.br.

Mariana Santos de Queiroz³⁴; Maria Luisa Paço-Larson

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto-USP, Ribeirão Preto – SP

A2BP1/FOX-1 is a member of a new family of RNA-binding proteins conserved in evolution. In *Drosophila melanogaster* the ortholog of FOX-1 is annotated as CG32062, which encodes five different isoforms generated from alternative splicing, according to *in silico* analysis based on a complete cDNA and EST data. We have examined by confocal microscopy the pattern of dFox-1 immunolocalization in the visual system of *Drosophila* during development. In the eye imaginal discs (ID) of third instar larvae, the label is mainly posterior to the morphogenetic furrow, which is a region of cell determination, proliferation and differentiation. In pupal retina the label follows a cell specific pattern. Label is restricted to the border of interommatidia cells of retina at 16% of pupal development (p.d.), and is no longer detected at 25% p.d. In the cone cells at 16-29 % p.d. the label seems to be on the cell border. In these cells at 33% p.d. labeling is intense and dispersed throughout the cytoplasm, but no longer detected at 37,5% p.d. In primary pigmentary cells the label in the cytoplasm is strong at 16-29 % p.d., diminishes at 33% p.d. and is absent at 37,5% p.d.. Markedly, heterogeneity occurs in the label pattern of primary pigmentary and cone cells; in the same ommatidia, strong labeled, weakly labeled and non-labeled cells can be observed. In the bristles, the label is first detected at 25% p.d., and is very weak at 37,5% p.d. In the cells of the ID or retina, dFOX-1 is concentrated in cytoplasmatic foci. Incubation of the ID in the presence of protein synthesis inhibitors, such as cycloheximide or puromycin, interferes in this pattern of dFox-1 sub-cellular localization in a way similar to that reported for protein components of RNA processing bodies (P-bodies). The localization of dFox-1 in cytoplasmatic foci was also disrupted in IDs treated with RNAase. Altogether, these data suggest that dFox-1 is involved in cellular process during the development of the compound eye of *D. melanogaster*, most probably acting in the control of translation.

Financial Support: FAPESP, CNPq, FAEPA-HCRP

MOLECULAR EVOLUTION OF THE GENE HOXA-13 IN SNAKES: WHAT IS THE FUNCTIONAL RELEVANCE OF CHANGES IN THE NUCLEOTIDE SEQUENCE?**Marina E. Singarete³⁵ and Tiana Kohlsdorf**

Department of Cellular and Molecular Biology, School of Medicine of Ribeirão Preto (FMRP) and Department of Biology, School of Philosophy and Sciences of Ribeirão Preto (FFCLRP). University of São Paulo, Ribeirão Preto, Brazil

³⁴ mariq_hc@hotmail.com

³⁵ marisingarete@usp.br.

Developmental genes are involved in multiple functions during embryo development, and present a clear fenotypic relationship as they determine the presence and position of several structures. Some of these genes are extremely conserved among species of vertebrates, as the gene family HOX. Although a relationship between the Hoxa-13 nucleotide sequence and autopodial anatomy is evident in a comparative analysis with more than 40 species of vertebrates, the functional relevance of mutations in the exon-1 region of this gene remains obscure in the context of the genetic network involved in limb formation in tetrapods. In this context, we aim to test the hypothesis that changes in the nucleotide sequence of the Hoxa-13 exon-1 that were identified in snakes as evolving under positive and directional pressure have a functional relevance and influence the expression of specific proteins, as Bone-Morphogenetic-Proteins (BMPs). We first need to standardize in our lab a system already developed for testing interactions between Hoxa-13 and BMP2 in mammals. Then, we construct recombinant plasmids with sequences of Hoxa-13 mutated in the five sites detected as evolving under directional selection in snakes. NG108-15 cells are transfected with these constructs, and interactions between Hoxa-13 and BMP2 are detected by Luciferase activity. The present study innovates for elucidating some of the possible functional effects of changes in nucleotide sequences in the cascade of molecular interactions involving Hoxa-13, focusing specifically on the expression Bone-Morphogenetic-Proteins.

Financial Support: Capes e FAPESP

IMMUNOCHARACTERIZATION OF DFOX-1 IN THE D.MELANOGASTER OVARY

***Mayara Terra Villela Vieira³⁶; Cirlei Aparecida Vanni Saraiva;
Maria Luisa Paçó Larson***

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de
Medicina de Ribeirão Preto - USP, Ribeirão Preto-SP

FOX-1 is an evolutionarily conserved protein with orthologs in metazoans. This family of proteins is characterized by the presence of a conserved RRM domain, indicating a functional relation with RNA metabolism. In agreement with this hypothesis, in vitro experiments have shown that in mouse and zebrafish these proteins can control tissue-specific alternative splicing. In *Drosophila*, the ortholog of FOX-1 (named here dFox-1) was annotated as CG32062 for which there are five isoforms predicted by in silico analyses and EST data. Results from our laboratory indicate dFox-1 as a possible component of mRNA processing bodies in S2R+ cells and in the eye-antenna imaginal discs and retina. Given the importance of proteins of mRNP complexes in the localization of maternal mRNAs in the oogenesis and initial embryogenesis, we have initiated a study on dFox-1 in the ovary of *Drosophila*. Here we present results on dFox-1 immunodetection using an antibody produced in our laboratory against a region that is common to all the predicted isoforms. Western Blot of protein extracts

³⁶ mayaratvv@gmail.com

of *Drosophila* ovaries probed with α -dFox-1 revealed three bands with molecular masses of about 82, 66 and 50 kDa, which are in the mass range of the isoforms predicted for dFox-1. Immunolocalization analysis by confocal microscopy showed that dFox-1 is concentrated at the posterior end of the developing oocyte in stages 3-7. In stage 8, it is detected along the oocyte cortex in the posterior, dorsal and ventral regions. In the nurse cells, the label is concentrated in the perinuclear region in stages 5 and 6, and accumulates in cytoplasmic foci in stage 7. In stages 8-10 dFox-1 label is very intense in the follicle cell layer of the egg chamber anterior region. Our data indicate the expression of at least three isoforms of dFox-1 in the *Drosophila* ovary. More interesting, some aspects of the sub-cellular localization of dFox-1 in the *Drosophila* egg chamber are similar to proteins such as dDCP1 and MeB31-2, which have a role in P-bodies and are also involved in mRNA localization in the oocyte.

Financial support: FAPESP, CNPq, FAEPA-HCRP

TRANSCRIPTIONAL ANALYSES OF CANDIDATE GENES RELATED TO CASTE DIFFERENTIATION IN *APIS MELLIFERA*

Mipsi Cristina Mori³⁷ and Klaus Hartfelder

Departamento de Biologia Celular e Molecular; Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto; Universidade de São Paulo; Ribeirão Preto, SP, Brasil

Social organization among bees is well characterized in *Apis mellifera* mainly with respect to caste differentiation which is induced by differential feeding in the beginning of third larval instar. In this process a cascade of endogenous reactions is triggered that influences genetic expression during development. Genome sequencing of *Apis mellifera* enabled the identification and annotation of genes associated to caste differentiation and two functional groups involved in regulation of metabolism and developmental processes were found in ESTs (Expressed Sequence Tags) clusters. Bioinformatics studies in experimental databases generated inferences about groups of genes co-expressed and their putative interaction. Five genes of these groups with the highest values of divergence regarding differential expression between queens and workers, during certain phases of development, and number of motives potentially regulated in upstream regions (5'-URL) were studied by transcriptional analyses. We aimed to validate the expression by Real Time PCR (qRT-PCR) of five genes inserted in these gene networks. As reference, larvae of workers and queens in two developmental stages were collected. The results obtained by qRT-PCR were analyzed by REST (Relative Expression Software Tool 2008, V2.0.7) software using queen phenotype as reference. In fourth larval instar of workers a significant higher expression of 13,5 fold was detected when compared to control for the predicted gene GB19006 (protein architecture was similar to domain DM9 in *Drosophila*). The *sesB* gene (stress sensitive B) presented 3,5 fold higher expression in fifth larval instar of workers. However, the gene *zeelin* did not present significant results. These data suggest that specially one predicted gene (GB 19006), functionally unknown, may be involved in caste differentiation.

³⁷ mybio2@usp.br.

Financial Support: CNPq and FAPESP

LACCASE 2 ENCODING GENE IN EXOSKELETON DIFFERENTIATION OF THE HONEY BEE, *APIS MELLIFERA* (HEXAPODA, HYMENOPTERA)

Moysés Elias-Neto³⁸1, Michelle P. M. Soares1, Zilá L. P. Simões1, Klaus Hartfelder2, Márcia M. G. Bitondi1

1 Departamento de Biologia; Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto; Universidade de São Paulo; Ribeirão Preto, SP, Brasil

2 Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos; Faculdade de Medicina de Ribeirão Preto; Universidade de São Paulo, Ribeirão Preto, SP, Brasil

The evolutionary success of insects is to a large extent due to the structural and mechanical properties of the integument, which is formed by the cuticle (or exoskeleton) and the subjacent epidermis. Building and maturation of the adult exoskeleton include complex biochemical pathways where the enzymes Laccases (E.C. 1.10.3.2) may have a key role. The function of these enzymes has been particularly linked to cuticle tanning (pigmentation and sclerotization). It was our aim to investigate the function and regulation of the gene encoding Laccase 2, *Amlac 2*, during development of the adult integument in the honeybee, *Apis mellifera*. RT-PCR analyses evidenced that *Amlac 2* is highly expressed in the integument of pharate adults in correlation with cuticle tanning. Consistent with the fact that exoskeleton differentiation occurs from the anterior to the posterior body axis, the increase in the levels of *Amlac 2* transcripts occurs earlier in the thoracic than in the abdominal integument. Post-transcriptional *Amlac 2* gene knock down resulted in abnormalities in cuticle structure, as revealed by histological analyses, and drastically affected the adult molt. Abdominal ligature in early pupae temporarily prevented the abdominal increase in the levels of ecdysteroids and *Amlac 2* transcripts, and severely impaired abdominal exoskeleton differentiation. Such results indicate that *Amlac 2* expression is controlled by ecdysteroids and has a critical role in the differentiation of the adult exoskeleton.

Financial Support: FAPESP.

IMMUNODETECTION OF LIPINS IN *DROSOPHILA*

Murilo Carlos Bizam Vianna³⁹, Paula Fernanda Gomes, Cirlei Aparecida Vanni Saraiva, Maria Luisa Paçó-Larson

Dept de Biologia Celular Molecular e de Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto

³⁸ meliasneto@pg.ffclrp.usp.br

³⁹ muvianna@yahoo.com.br.

Lipin belongs to a new family of evolutionarily conserved proteins that present phosphatidic acid phosphatase Mg^{2+} dependent activity, acting in biosynthesis of storage lipids and membrane phospholipids, and as a transcriptional co-activator of lipogenic genes in mammals. In the present work we developed antibodies against two fragments of the *Drosophila melanogaster* lipin and characterized its sub-cellular localization in S2R+ cells. In immunoblots of protein extracts from S2 cells both antibodies detected a major band of ~130 kDa, which is in agreement with the masses of the polypeptides deduced from the lipin cDNAs. Immunostaining of lipin in S2R+ cells reveals a cytoplasmic dispersed localization; with a small percentage of cells (~5%) presenting the label apparently concentrated in the periphery of vesicles with maximum diameter of 3 μm , and devoid of neutral lipid. Biochemical experiments showed that most of the lipin proteins are soluble in S2 cells, although a small fraction co-sediment with heavy membranes. This observation, and the fact that only a small percentage of cells presented lipin concentrated in vesicles raise the possibility that the lipin-positive vesicles might reflect a dynamic process potentially related to the biogenesis of membranes needed for growth before the cell division.

Financial Support: CAPES, CNPq, FAPESP, FAEPA

EXPRESSION PATTERN OF GENES RELATED TO LIPID RAFTS FORMATION DURING XENOPUS LAEVIS DEVELOPMENT

***Nathália da G. Amado⁴⁰; Débora M. Cerqueira;
and José Garcia Abreu***

Programa de Biologia Celular e do Desenvolvimento - ICB - UFRJ

Lipid rafts are membrane microdomains (MM) with high content of cholesterol and sphingolipids. It has been shown that these structures play a role in intracellular trafficking and can act as platforms for signaling complex. Recently we showed that cholesterol depletion within plasma membrane disrupted MM assembly, affecting anterior development of *Xenopus* embryos. These data suggest a correlation between cholesterol-rich MM and the signaling pathways required for head formation. Two genes previously identified and related with cholesterol biosynthesis (Xen 2) and organization of lipid rafts (Xen 1) are here investigated.

Since cholesterol and MM seems to play a role during *Xenopus* development, we decided to investigate the expression pattern of these genes during embryonic development of *Xenopus laevis*.

The cDNAs containing ESTs (expression sequence tag) of these genes were obtained at gene database. These sequences showed high degree of identity with human, zebra-fish, rat and mouse, and are therefore possible candidates for orthologues in *Xenopus*. In situ hybridization was performed in different stages of development and showed these genes are expressed at the animal pole, branchial arches, optical vesicles, olfactory placodes, pronephric area and neural plate. At late neurula stage Xen2 expression was restricted in neural crest

⁴⁰ nathalia@anato.ufrj.br

tissue. At tailbud and tadpole stages all of them showed expression at dorsal region, along the anteroposterior axis and somites.

The PCR analysis revealed Xen1 and 2 are maternally expressed and can also be detected at latter stages. Interestingly, Xen1 expression was dramatically reduced at stages 20, 25 and 30, suggesting that this gene need to be finely regulated during *Xenopus* development.

These results showed that Xen1 and Xen2 are expressed at restricted areas and specific embryonic stages, suggesting that these ortologues can play a significant role during *Xenopus* development. Overexpression and functional studies are under investigation.

FUNCTIONAL VALIDATION OF POTENTIAL RING GLAND ENHANCERS IDENTIFIED IN THE *DROSOPHILA MELANOGASTER* GENOME

***Patricia Vieira Zemp⁴¹, Simone Sakagute Tavares
and Nadia Monesi***

Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil

The BhC4-1 gene is expressed in the salivary glands of *Bradysia hygida* at end of the fourth larval instar. The mechanisms that regulate the BhC4-1 salivary gland expression are conserved in transgenic *Drosophila*. Functional studies revealed that the BhC4-1 expression also occurs in the ring gland of transgenic *Drosophila* and led to the identification of a 67 bp ring gland enhancer. A bioinformatics analysis identified 67 sequences in the *D. melanogaster* genome that are similar to sequences contained in the ring gland enhancer. Further bioinformatics analysis revealed that 10 out of the 67 sequences are localized in potential regulatory regions, are conserved in at least 7 *Drosophila* species. In addition, for these 10 sequences, the available data for the nearest downstream open reading frame is inconclusive with respect to embryonic expression.

In order to functionally validate part of these sequences, the genomic sequences localized in the regulatory regions of CG12433, CG13711, CG15306 and CG10609 were cloned into the pBSKS+ vector. The regulatory regions of CG12433, CG13711 and CG15306 were transferred to the *Drosophila* P element vector, pBP. The constructs present the following structure: P element / white / regulatory region to be tested / Fbp1 basal promoter / lacZ / P element. The constructs CG12433-pBP and CG13711-pBP were both injected into *D. melanogaster* embryos and transgenic lines were obtained. The pattern of expression of the reporter gene has already been analyzed in five transgenic lines transformed with the CG12433-pBP construct. The results indicate that the tested fragment does not drive ring gland gene expression. These results are not totally unexpected since data derived from a bioinformatics analysis, designed to identify genes that are potentially regulated by the transcription factor Dorsal in *D. melanogaster*, led to the demonstration that only about one third of the identified Dorsal binding sites present functional relevance. In order to extend the functional validation of part of the sequences identified through the bioinformatics analysis, in the near future we plan to

⁴¹ patriciavz@yahoo.com.br.

investigate the pattern of expression driven by the cloned genomic fragments localized in the regulatory regions of the CG13711, CG15306 and CG10609.

Financial support: FAPESP, CNPq and PRP-USP.

DROSOPHILA MELANOGASTER LIPIN PRESENTS DISTINCT TISSUE AND DEVELOPMENTAL EXPRESSION PATTERNS

Rafaela Martins Maia⁴², Valéria Valente, Murilo Carlos Bizam

Vianna and Maria Luisa Paçó-Larson

Department of Cellular and Molecular Biology Pathogenic Bioagents, Faculty of Medicine of Ribeirão Preto, University of São Paulo; São Paulo, Brazil

Lipins encompass a novel family of evolutionarily conserved proteins recently characterized as Mg²⁺-dependent phosphatidate phosphatases (PAP1), with a role in the biosynthesis of membranes and lipid metabolism. While a single lipin gene is detected in less complex organisms, such as yeast and worm, in mammals there are three genes that express different lipin paralogs in a tissue-regulated manner. We show that the unique ortholog of lipin identified in the *Drosophila* genome (CG8709) expresses at least three isoforms, generated by alternative splicing and transcription initiation, nominated here as DmLpinA, DmLpinB and DmLpinC. Their expression is regulated at the mRNA and protein levels during development and in the different tissues. The amounts of lipin mRNAs are higher in embryos and male adults than in larvae, while lipin proteins are more abundant in third instar larvae. Moreover, while the level of DmLpinA mRNA is higher in extracts from the intestinal tract/Malpighi tubules, the amount of lipin detected on immunoblots of protein extracts is relatively low. On the other hand, although lipin proteins are present in elevated amounts in protein extracts of the fatty body, the levels of DmLpinA and B mRNAs detected in this tissue are lower than in the other analysed tissues. DmLpinC isoform is exclusively expressed in testis. In addition to the DXDXT motif characteristic of PAP1, the *Drosophila* lipins present a conserved nuclear localization signal and the LXXIL coactivator motif that in mammals are essential for the nuclear activity of these proteins, suggesting that *Drosophila* lipins might have both a cytoplasmatic and nuclear function. In agreement with this hypothesis, confocal microscopy immunolocalization analysis, using an antibody that recognizes all *Drosophila* lipin isoforms, detected label in the cytoplasm and nuclei of third instar larvae fat cells. In whole mount preparations of intestinal tract/Malpighi tubules, lipin was detected exclusively in the cytoplasm of midgut longitudinal muscle cell layer; similarly, in the testis it was also restricted to the muscle layer. In both cases the label presented a sarcomeric pattern, indicating that lipin is concentrated in the A band. Also in the ovaries, the pattern of lipin subcellular label was peculiar, being heavily concentrated in the ring channels, which make connections between the cytoplasm of nurse cells and the oocyte.

⁴² rafaela.rafam@gmail.com.

Financial support: FAPESP, CNPq, FAEPA-HCRP

RHO-ROCK SIGNALING IS NECESSARY FOR LENS PLACODE INVAGINATION IN CHICK EMBRYOS

***Ricardo Moraes Borges⁴³, Marinilce Fagundes dos Santos
and Chao Yun Irene Yan***

Dept de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas da
Universidade de São Paulo, São Paulo, SP

Vertebrate lens is formed by invagination of the placode that overlies the optic vesicle. Here, we investigated the contribution of acto-myosin cytoskeleton and the Rho-ROCK pathway to the lens placode initial invagination. Actin filaments and myosin II were detected at the apical region of chick lens placode prior to invagination whereas no such polarization was detected in the surrounding non-lens ectoderm. Inhibition of actin polymerization and myosin II ATPase activity with Cytochalasin D or Blebbistatin treatments, respectively, completely abolished lens placode invagination, indicating the relevance of actin-myosin contraction to the initial lens placode bending step. RhoA, an acto-myosin regulator is also apically enriched in the lens placode prior to its bending. Inhibiting Rho signaling by C3 exoenzyme electroporation or its downstream effector Rock by Y27632 treatment also arrested lens placode invagination. Moreover, we show that inhibition of Rock also reduced the concentration of actin filaments and myosin II at the lens apical side. Taken together, these data suggest that contraction of the apical acto-myosin in the lens placode might initiate its invagination and that the Rho-Rock pathway is necessary for the correct localization of acto-myosin cytoskeleton at the lens placode apical portion.

Financial Support: FAPESP and CNPq

HYPOXIA PATHWAY AS A PUTATIVE NOVEL ELEMENT IN THE HONEY BEE CASTE DETERMINATION PROCESS

Sergio Vicente de Azevedo⁴⁴, Tatiane Lippi², and Klaus Hartfelder²

1Depto. Genética, FMRP-USP 2Depto. Biologia Celular e Molecular e Bioagentes
Patogênicos, FMRP-USP, Ribeirão Preto

Honey bees (*Apis mellifera*) provide a prime example of diphenic development. Females develop into either of two castes, queen and workers, that differ not only in size and several structural features but also in individual fitness and life history. Developmental fate is initially determined environmentally, by the larval diet, which then acts on two important regulators of

⁴³ borgesrm@gmail.com.

⁴⁴ svazevedo@rge.fmrp.usp.br.

caste determination, juvenile hormone and the insulin/TOR pathway. Recent studies on *Drosophila melanogaster* suggest a third possible mechanism that may play a role in this process, the hypoxia pathway which is controlled by transcription factors of the helix-loop-helix-PAS (HLH-PAS) family. In order to obtain information on this pathway in honey bees we used, as a first approach, available genomic information from *Drosophila melanogaster* to find the respective ortholog genes in *Apis mellifera*. BLAST searches on the honey bee genome database revealed three genes which showed best matches with the *Drosophila* hypoxia genes (*sima*, *fatiga* and *tango*), and these were named *amsima*, *amfatiga*, *amtango*, respectively. The three orthologs were sequenced, annotated by Artemis v9.0 and their expression profiles were checked by RT-PCR in postembryonic stages of worker bees. Our analysis showed that *amsima* is expressed almost without variation from the fourth larval instar until the end of the prepupal stage, while *amtango* and *amfatiga* showed a higher expression in the cocoon-spinning stage. Taken together, this focus on the hypoxia pathway rekindles interest in yet little understood studies performed in the 1980s which had suggested differences in respiratory metabolism of queen and worker larvae. The genomic information and quantification of expression levels of hypoxia genes now allows to follow these earlier clues and to investigate a novel element in the complex process of diphenic development in this social insect.

DEVELOPMENTAL MECHANISMS UNDERLYING CARDIAC ANTERO-POSTERIOR PATTERNING BY THE RALDH2 CAUDOROSTRAL WAVE

Sylvia Sura Trueba⁴⁵ and José Xavier-Neto

Grupo de Genética e Desenvolvimento Cardíaco, Laboratório de Genética e Cardiologia Molecular, InCor, HC/FMUSP

Early establishment of anteroposterior (AP) orientation of cardiac precursors is a key step in heart organogenesis because anterior and posterior segments give rise to cardiac ventricles and atria, respectively. Previous findings from the laboratory indicated that a wave of *Raldh2* promotes asymmetric synthesis of retinoic acid (RA) to the cardiac field in the AP axis. This caudorostral wave endows the posterior cardiac mesoderm with the capacity to synthesize its own RA, which instructs this progenitor area to differentiate as sinus and atrial tissues.

We cultured HH5-6 chicken embryos on albumin plates overnight at 37° and observed that they were able to develop to HH7-8, expressing the normal *raldh2* caudorostral wave invading the cardiac field. Afterwards, we cultured isolated cardiac mesoderm fragments (CMF) in the same conditions and found that most of these fragments were unable to express *raldh2* (93%). We then cultured CMF from chicken with its corresponding posterior region from quail to try and restore the *raldh2* caudorostral wave. We performed a QcPN Immunohistochemistry *in toto* to differentiate quail from chicken tissue. In these culture conditions, we restored the *raldh2* expression in 35% of the CMF, i.e., five times more than in isolated fragments, without significant migratory contribution of quail cells to the anterior domain of *raldh2* expression. Surprisingly, quail axial tissue, normally unable to induce *raldh2* expression, restored in 51% the *raldh2* caudorostral wave when juxtaposed to the chicken CMF, suggesting a non-specific

⁴⁵ sylvia.sura@gmail.com

reestablishment of *raldh2* expression in these culture conditions. To test the development of *raldh2* caudorostral wave in the cardiac field, disassociated from the posterior mesoderm, in the whole embryo; we are currently cultivating, for 3, 6 or 12 hours, HH5-6 embryos where the left-side posterior and cardiac mesoderms are separated by a slide of glass. Longer times of incubation brought morphological disturbances, sometimes including cardia bifida. It remains to be confirmed if *raldh2* expression in these experiments is perturbed and in which manner to further explore the possible candidates as regulators of *Raldh2* transcription.

Financial Support: FAPESP

FORMATION OF THE GERMINAL EPITHELIUM DURING THE GONADAL MORPHOGENESIS IN MALE OF THE CYPRINUS CARPIO (TELEOSTEI: OSTARIOPHYSI: CYPRINIFORMES)

Talita Sarah Mazzoni⁴⁶1,2 e Irani Quagio-Grassiotto2

1 Programa de Pós-Graduação em Biologia Celular e Estrutural, Instituto de Biologia, UNICAMP, Campinas, SP

2 Depto de Morfologia, Instituto de Biociências de Botucatu, UNESP, Botucatu, SP

During the gonadal morphogenesis in *Cyprinus carpio* the proliferation and differentiation of germ and somatic cells from the gonadal primordium result in the formation of testicular structures and the constitution of germinal epithelium that borders seminiferous tubules. Thus, to study these events, gonadal tissues of juveniles of *Cyprinus carpio* were prepared for observation under transmission electron microscopy and high resolution light microscopy, including Reticulin's Method.

The gonadal primordium is an elongated structure with individual primordial germ cells (PGCs) scattered among somatic cells. In the undifferentiated gonad, somatic cells wrap around and individualize the PGCs. PGCs divide and form continuous cords delimited by a basement membrane. Reorganization from PGCs results in the loss of the cord organization. Each PGC, now differentiated in spermatogonia, is wrapped, by the now pre-Sertoli cells giving rise to a cyst. The cysts join one another forming acinar-clusters, the first signal of the male gonad differentiation. A basement membrane is synthesized around each cluster, being incomplete between adjacent clusters. Pre-Sertoli cells rest upon the forming basement membrane.

In the center of the clusters a space is created when Sertoli cells move away from one another. In the region where the basement membrane is absent, nearby clusters fuse to one another becoming connected by the same luminal space. The progressive fusion of the clusters gives rise to the seminiferous tubules that are bordered by the newly formed germinal epithelium constituted by the cysts that rest upon the basement membrane. Mesenchymal cells surround the seminiferous tubules give rise to the cellular components of the interstitial compartment.

⁴⁶ talitasarah@yahoo.com.br.

Inside the cysts spermatogenesis starts. As spermatozoa is releasing, the anastomosis of the testicular tubules occurs forming the spermatic duct on the dorsal region of the testis. During all gonadal development the basement membrane separates the germinal epithelium from the interstitial compartment.

Financial Support: CNPq, CAPES/PROEX.

MYOSIN VA EXPRESSION DURING EARLY SENSORY NEUROGENESIS

Tatiane Kanno⁴⁷, Leon Maas, Enilza Espreafico[#] e C.Y. Irene Yan

Dept de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas,
USP, São Paulo, SP

[#] Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, USP,
Ribeirão Preto, SP

Dorsal root ganglia (DRG) are components of the peripheral nervous system that harbor the cell bodies of sensory neurons. During normal development, the establishment of the appropriate connections through neuritogenesis is an essential step in sensory neurogenesis. In this process, regulation of growth cone directionality and speed contribute towards the guidance and control of filopodial extension. Myosin Va, an evolutionarily conserved molecular motor involved in organelle transport, has been implicated in filopodial extension. Myosin Va is widely expressed in the vertebrate developing nervous system. The inactivation of Myosin Va in growth cones of chick dorsal root ganglion neurons in culture significantly decreases the rate of filopodial extension. Moreover, mouse mutants for Myosin Va present severe neurological defects, which phenocopy human Griscelli's Syndrome. Taken together, those findings suggest that Myosin Va plays an important role in neural development. However, since previous studies on its role in neuritogenesis have been carried out *in vitro*, we aim to assess the role of Myosin Va in sensory neuritogenesis *in vivo*. Thus, we first characterized the expression of Myosin Va at different stages of DRG development: at the start of its condensation (HH19), at the onset of neuronal production (HH25) and after the appearance of the ventral root (HH30). Furthermore, we correlated the expression of Myosin Va with that of Islet-1. Islet-1 is a transcription factor expressed in post-mitotic DRG neuronal cells and a known marker of early neuronal differentiation. Our results show that Myosin Va is expressed exclusively in Islet 1-positive cells in the DRG of all stages investigated. Islet-1 positive cells in the HH19 DRG do not present discernible neurites. Thus, the expression of Myosin Va in cell bodies at this stage suggests that Myosin Va might contribute towards other events in early neurogenesis and/or is required for the initiation of neurite extension. As DRG development progresses, Islet-1-positive cells emit neurites that label positively for Myosin Va, thus confirming the previous reports on its role in sensory neuritogenesis. Taken together, the data presented here suggest that Myosin Va might contribute not only towards sensory neuritogenesis but also towards other events in early neurogenesis.

⁴⁷ kannotatiane@gmail.com.

Financial support: FAPESP and CNPq

ORGANIZATION AND LARVAL TRANSCRIPTION PROFILE OF GENES ASSOCIATED TO NEUROGENESIS IN *APIS MELLIFERA* CASTES

Vanessa Bonatti⁴⁸¹; Ana D. Bomtorin²; Zilá L.P. Simões³; and Angel R. Barchuk^{#1}

1Depto. Ciências Biomédicas, Universidade Federal de Alfenas, Unifal-MG. 2Depto. Genética, Universidade de São Paulo-FMRP. 3Depto. Biologia, Universidade de São Paulo-FFCLRP, Brazil

Contrary to the intuitive thinking, dietary restriction has been increasingly shown as a key trigger of neurogenic events in several model systems. In the honeybee *Apis mellifera*, females that receive low amount of food during larval development become workers, bees with a proportional more developed adult brain than that of the higher fed queens. A more developed brain allows workers to accomplish the variety of duties needed to maintain a healthy colony: foraging, nursing, nest cleaning and protecting, etc. During most part of post-embryonic development, however, queen's brain shows a bigger area of neuroblasts than that of workers, what may reinforce the intuitive idea of the importance of a high nutritional input as responsible for a high nervous system output. Extensive cell death events during the second part of pupal development turn queen's brain proportionally less developed than that of worker's. Due to the differential feeding on presumptive queen and worker larvae, the early larval differential neurogenesis in honeybees is a natural experiment and good model to be used for the identification of genes linking differential nutrition to differential nervous system development. Here, we report the organization and larval transcription profile in queens' and workers' honeybee brain of seven genes known as associated to neurogenesis in different animal models and previously found as differentially expressed between castes in the whole body of fourth instar larva honeybees (Barchuk et al., BMC Dev Biol, 2007): tetraspanin 5D (tsp5D), dachshund (dac), failed axon connection (fax), cryptocephal (crc), ataxin-2 (atx-2), Ephrin Receptor (ephR) and short stop (shot). Using qPCR we analyzed the expression profile of these genes in brains of third to fifth larval stages. Our results show three groups of expression patterns: One including profiles with expression levels increasing progressively, represented by the expression of dac and fax; one with profiles peaking in fourth instar larvae, represented by atx-2, EphR and shot; and the last one represented by tsp5D, showing a decreasing profile. Shot, whose expression is required for the axons extension and cell proliferation in *Drosophila*, was found more expressed in fourth stage queen larvae. This may help explain the existence of a larger area of neuroblasts in brains of fifth instar queen larvae when compared to workers and indicates shot as a pivotal player in the scene of the gene expression cascade induced by differential feeding in animals, particularly in the honeybee *A. mellifera*.

⁴⁸ nessabonatti@hotmail.com #barchuk@unifal-mg.edu.br.

Financial Support: CNPq Processo No 473748/2008-8

GENERATION OF MUTANTS FOR THE CALPAIN A LOCUS IN DROSOPHILA MELANOGASTER USING P ELEMENT AS MUTAGENIC AGENT

Viviane Vieira⁴⁹ and Helena Araujo

Institute for Biomedical Sciences – UFRJ, Rio de Janeiro, RJ – Brazil

In early embryonic development, a gradient of nuclear protein NFκB / Dorsal is required to establish the ectoderm and mesodermal domains. Maternally expressed genes encode components of a proteolytic cascade that culminates in activation of the Toll transmembrane receptor, inducing degradation of the inhibitor IκB / Cactus and allowing nuclear translocation of Dorsal protein. Parallel to the Toll pathway, signaling through maternal BMP4/Dpp inhibits the degradation of Cactus, regardless of the signal from Toll. Studies in our laboratory (Fontenele, M et al, 2009) describe a new component in this pathway, the protease Calpain A (CalpA). CalpA alters Cactus levels, while mutant loss-of-function for cact alter the activity of CalpA. To investigate in more detail the interaction between these two genes and the effects of CalpA on embryogenesis, we aimed to generate mutants for the CalpA locus. The strategy is based on the use of a transposing element located in the promoter region of the gene CalpA, that when mobilized may delete some sequences of this gene and compromise its expression. With this methodology we established 20 homozygous lethal lines which showed excisions of the transposing element. All lines presented similar phenotypes: anterior holes in the cuticle and lowered staining with CalpA antisera. These phenotypes are consistent with decreased zygotic expression of CalpA and suggest that this locus contributes to the establishment of anterior structures of the larva. Complementation tests show that almost all lines do not complement and thus represent alleles of a same locus. However, lethality was complemented by two deficiencies that cover the CalpA locus. In order to understand these contradicting results we are characterizing in more detail these deficiencies and their resulting embryonic phenotypes.

Financial Support: CNPq FAPERJ

CHARACTERIZATION OF MADS-BOX GENE FAMILY IN COFFEA ARABICA

Raphael Ricon de Oliveira⁵⁰ and Marcelo Carnier Dornelas

UNICAMP – Instituto de Biologia, Dep. Biologia Vegetal, Rua Charles Darwin s/no

⁴⁹ vivianevox@hotmail.com .

⁵⁰ rapharicon@gmail.com

The MADS-box gene family encodes transcription factors that act as key regulators in many steps in the development of various organisms. In plants these genes are involved in determining the identity of reproductive meristems and floral organs, in the control of flowering time, in fruit development, among other process. This study aims to identify genes of MADS-box family related to the determination of floral organ identity in *Coffea arabica*. Searches were conducted by similarity between MADS sequences deposited in GenBank and expressed sequence tags (EST) database arising from The Brazilian Coffee Genome Project. Bioinformatics programs were used for assembly the sequences in EST-contigs, that after resequencing their representative clones, could be confirmed. In this phase, polymorphisms between alleles could be observed, besides differences in splicing expression. A phylogenetic analysis was made indicating putative orthologs of genes described and studied in *Arabidopsis thaliana*, allowing us to establish a starting point to test this hypothesis by comparing the expression profiles in different tissues and stages of reproductive development.

CHARACTERIZATION OF EXPRESSED GENES DURING EMBRYOGENESIS IN PASSIFLORA EDULIS

Juliana Lacorte Cazoto⁵¹ and Marcelo Carnier Dornelas

Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Cidade Universitária "Zeferino Vaz", Barão Geraldo, CEP: 13418-970, Campinas, SP

Different from animal development, that has its organs formed during the embryo development, plant structures are formed by the shoot and root apical meristems (SAM and RAM, respectively), which are established during plant embryogenesis. This process is organized by the concomitant activity of specific transcription factors that are expressed in a hierarchical way, determining cellular domains that are responsible for the installation and maintenance of these meristems. The activity of those transcription factors is under control of environmental information like light, hormonal concentrations among others. With the aim of characterizing the embryo development in the commercial passion fruit, *Passiflora edulis* Deg, we identified and characterized genes expressed during the zygotic and somatic embryogenesis. Among the identified genes, members of NAC/CUC, YABBY, WOX, SCARECROW, AUX/IAA and ARF families were found. These genes were identified by bioinformatics analysis in the data bank from PASSIOMA project. The expressed sequence tags (ESTs) were clustered and the predicted protein sequences compared by sequence alignment with their potential homologs in *Arabidopsis*. The gene expression pattern will be studied by in situ hybridization during different stages of *P. edulis* embryo development.

Funding Agency: FAPESP (Proc. 08/57588-1)

⁵¹ jucazoto@gmail.com.

ANATOMICAL CHARACTERIZATION OF THE ANDROGYNOPHORE MOVEMENT IN PASSIFLORA SPP. SECT. XEROGONA I. PASSIFLORA CITRINA

Livia Scorza⁵² and Marcelo Carnier Dornelas

Universidade Estadual de Campinas, Instituto de Biologia, Departamento de Biologia Vegetal. Cidade Universitária "Zeferino Vaz". Campinas, SP

Some species of Passiflora Subgen. Decaloba, sect. Xerogona (Raf.) Killip exhibit a touch-induced movement of the androgynophore. This reaction is a relatively rapid response in a direction determined by the placement or direction of the stimulus. The androgynophore movement in Passiflora is not yet described in the literature and remains as an anecdotal observation of collectors of ornamental species from this gender. This androgynophore particular behavior could be related to pollination habits of these species. Thus, the aim of this work is to characterize the androgynophore movement through an anatomical study of one of the species from section Xerogona, Passiflora citrina. The samples were collected after being stimulated or not by touch, fixed in 4% paraphormaldehyde, dehydrated in an ethanol series and embedded in plastic resin glycol methacrylate. The materials were cut in a rotative microtome, stained with toluidine blue and the permanent slides were prepared for optical microscope observation. Our partial results show that the androgynophore movement is probably caused by changes in the cortical cells volume, on the base of the androgynophore column, in the stimulated side. In transverse sections it was observed that, in approximately one third of the circumference, stronger stained extracellular content and more plasmolized cells as compared to the cells of other regions. This means that the decrease in the cells volume is due to water loss and of other still uncharacterized compounds. We hope to provide a better understanding of pollination systems evolution in Passiflora and of the floral morphology dynamics of these species and their pollinators.

CHARACTERIZATION OF MADS-BOX GENES FROM CITRUS INVOLVED IN FRUIT DEVELOPMENT

Pedro Araújo⁵³ e Marcelo Carnier Dornelas

UNICAMP – Instituto de Biologia, Dep. Biologia Vegetal, Rua Charles Darwin s/no

Some molecular elements involved in fruit development are extensively studied in Arabidopsis thaliana – a model plant, which produce dry fruits called siliques. The FRUITFULL (FUL) and SHATTERPROOF (SHP), that belong to multigenic family MADS-box, are related to ontogenesis and ripening of Arabidopsis dry fruit. Recent studies suggest that these genes, FUL and SHP, are conserved in dry and fleshy fruits. Thus, we decided to characterize, minutely, the development of Citrus sinensis e Citrus reticulata and identify

⁵² livia@scorza.com.br.

⁵³ paraujo@unicamp.br / araujo.pedro@gmail.com.

possible homologies FUL e SHP in Citrus spp. Furthermore, the aim of this study was to evaluate the expression pattern of these homologies during fruit development of *C. sinensis*. The data bank of project CitEST contains 36 genes homologous to MADS-box genes, six of which correspond to FUL and SHP in four species of Citrus. These sequences were characterized by bioinformatic tools and phylogenetic analysis. The use of light and scanning electron microscopy (SEM) techniques allowed the identification of important anatomic differences in mesocarp and exocarp during fruit development in oranges and tangerines. RT-PCR and in situ hybridization results showed the expression pattern of FUL and SHP genes through fruit development in Citrus.

5-BROMO-2'-DEOXYURIDINE INHIBITS THE ACTIVITY OF THE AMPLIFIED GENES, BHC5-2, BHC4-1 AND BHB10-1, IN THE SALIVARY GLAND OF BRADYSIA HYGIDA (DIPTERA, SCIARIDAE)

Juliana Conti⁵⁴ Viana e Jorge Cury de Almeida

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto, SP

Among the several effects of the 5-bromo-2'-deoxyuridine (BrdUrd), an analog of thymidine (dThd), the inhibition of cell differentiation is the most interesting. The injection of BrdUrd into *Bradysia hygida* larvae, during the period of gene amplification, leads to a notable morphological result – the DNA puffs do not expand, the chromatin, in these chromosomal sites, remains very compacted during all the rest of the 4th larval instar and transcription process seems to be severely decreased in the DNA puffs.

Here we show, by morphological and molecular techniques, the effect of BrdUrd on cell differentiation, studying its role on the activity control of three specific amplified genes: *BhC5-2*, *BhC4-1* and *BhB10-1*.

In the experiments, 1 µl of 4 mM solutions, of dThd (control) or BrdUrd, was injected into larvae, during the first part of the gene amplification period. We performed Total RNA extraction, RT-PCR or Northern blotting followed by nucleic acid hybridization. Protein synthesis was studied by SDS-PAGE, Western blotting and immuno-detection.

The treatment with BrdUrd had a very pronounced inhibitory effect on mRNA production, demonstrated by RT-PCR (for gene *BhC5-2*) and Northern blot (for genes *BhC4-1* and *BhC5-2*). The same occurred in relation to the synthesis of proteins *BhC4-1* and *BhB10-1*. These experiments show an interesting correlation, between the morphological effect of BrdUrd on the DNA puff anlagen, and the activity of specific amplified genes. Next step, comparison with the activity of non-amplified genes will be established.

Financial Support: CAPES, FAPESP e FAEPA.

⁵⁴ juconti@usp.br

ABSTRACTS PRESENTED IN - X BRAZILIAN SYMPOSIUM ON EXTRACELLULAR MATRIX AND V INTERNATIONAL SYMPOSIUM ON EXTRACELLULAR MATRIX

PROGRAMA AT A GLANCE

Nov 1	18h00		Opening ceremony
	18h30	Conference	The metastatic niche: adapting the foreign soil
Nov 2	8h30	Symposium 1	ECM and maternal- fetal interface
	10h30	<i>Coffee break</i>	
	11h00	Symposium 2	ECM interplay with soluble proteins: role in cell migration
	13h00	<i>Lunch/ free time</i>	
	15h00	Course	3D model for cell culture
	16h00	Symposium 3	ECM and cancer cell biology
	17h30	Conference	Cell communication in tumoral microenvironment
	18h30	<i>Coffee break</i>	
	19h00		Poster Session I
Nov 3	8h30	Symposium 4	Glycosaminoglycans: a whole world in ECM biology
	10h30	<i>Coffee break</i>	
	11h00	Symposium 5	ECM in tissue differentiation/dedifferentiation and pathology
	13h00	Lunch/free time	
	14h15	Course BD Conference	3D model for cell culture Aplicações das matrizes extracelulares: Matrigel™ e o novo PuraMatrix™ para Cultura 3D

	16h00	Symposium 6	ECM in chronic/degenerative diseases
	17h30	Conference	Biomechanical and signaling roles of extracellular matrix in regulating early morphogenesis
	18h30	<i>Coffee break</i>	
	19h00		Poster Session II
Nov 4	8h30	Symposium 7	ECM and stem cell biology and therapy
	10h00	<i>Coffee break</i>	
	10h30	Conference	Signaling pathways involved in the effect of heparin on endothelial cells
	11h30		Closing ceremony

DETAILED PROGRAM

November 1st

18h00 Opening Ceremony

18h30 Opening Conference

Chairperson: Roger Chammas

The metastatic niche: adapting the foreign soil

David Lyden (Cornell University, USA)

November 2nd

8h30- 10h30 Symposium 1: ECM and maternal-fetal interface

Chairperson: Telma Zorn

08h30- 09h20 Regulation of trophoblast cell behavior by extracellular matrix

Ann Sutherland (Univ. Virginia Health System, USA)

09h20- 09h50 Extracellular matrix remodeling at the maternal-fetal interface

Telma M.T. Zorn (USP, Brazil)

09h50-10h15 Hormone-regulated expression and distribution of versican in mouse uterine tissues

Renato de Mayrinck Salgado (USP, Brazil)

10h15- 10h30 Selected abstract: Expression of decorin and biglycan in human normal term placenta and in invasiveness- changed trophoblast pathologies

Alexandre Urban Borbely (USP, Brazil)

10h30 coffee break

11h00- 13h00 Symposium 2: ECM interplay with soluble proteins: role in cell migration

Chairperson: Cecília Hedin Pereira

11h00- 11h45 The Extracellular Matrix as a Modulator of Tumor Invasion and Metastasis

William G. Stetler-Stevenson (NIH, USA)

11h45- 12h30 ECM and soluble proteins control neuronal cell migration

Cecilia Hedin Pereira (UFRJ, Brazil)

12h30-12h45 Selected abstract: April modulates in vitro thymocyte migration and cell adhesion by interacting with HSPG

Cecília Rocha (FIOCRUZ, Brazil)

12h45-13h00 Selected abstract: The role of laminin and CXCL12 in T-cell migration during cardiac allogeneic graft rejection

Ingo Riederer (FIOCRUZ, Brazil)

13h00 15h00 lunch/free time

15h00 -16h00 Course: 3D Model for Cell Culture

Vanessa M Freitas (USP, Brazil)

16h00- 17h30 Symposium 3: ECM and cancer cell biology

Chairperson: Ruy Jaeger

16h00-16h30 Laminin peptides influencing carcinoma behavior

Ruy Jaeger (USP, Brazil)

16h30- 17h00 Platelet activation as a novel target for galectin-1-glycan interactions: implications in inflammation, thrombosis and metastasis

Mirta Schattner (ANM, Argentina)

17h00-17h15 Selected abstract: RECK-mediated inhibition of glioma migration and invasion

Renato Ramos Massaro (USP, Brazil)

17h15-17h30 Selected abstract: The RECK B isoform is downregulated by simvastatin in human melanoma cells

Fernanda Augusta de Lima Barbosa (UFPR, Brazil)

17h30- 18h30 Conference

Chairperson: Mauro Pavão

Cell communication in tumoral microenvironment

Lubor Borsig (Univ. Zurich, Zwitzerland)

18h30 19h00 coffee break

19h00- 21h00

Poster Session I

November 3rd

8h30- 10h30 Symposium 4: Glycosaminoglycans: a whole world in ECM biology

Chairperson: Paulo Mourão

08h30- 09h15 Defining the heparan sulfate code that increases FGFR function during branching morphogenesis

Matt Hoffman (NIH, USA)

09h15-10h00 Sulfated polysaccharides ensures a carbohydrate-based mechanism for species recognition during sea urchin fertilization

Paulo Mourão (UFRJ, Brazil)

10h00-10h15 Selected abstract: Structural characterization and anticoagulant properties of a unique heparan sulfate from a mollusk bivalve

Angélica Maciel Gomes (UFRJ, Brazil)

10h15-10h30 Selected abstract: Fucan and the stimulus in the synthesis of antithrombotic endothelial heparan sulfate: relationship between structure and biological activity

Valquíria P Medeiros (UNIFESP, UFRN, Brazil)

10h30- 11h00 coffee break

11h00- 13h00 Symposium 5: ECM in tissue differentiation/dedifferentiation and pathology

Chairperson: Eva Burger

11h00-11h45 Vascular Extracellular matrix and Vessel Wall Development

Robert Mecham (Washington Univ, USA)

11h45-12h30 ECM in experimental paracoccidiosis infection

Eva Burger (USP, Brazil)

12h30-12h45 Selected abstract: Laminin alpha5 expression after human myoblast transplantation into Rag-/- Gammac-/- mice

Ingo Riederer (FIOCRUZ, Brazil)

12h45-13h00 Selected abstract :Schwann cells transdifferentiation: possible role of myofibroblast in neural fibrosis during leprosy

Rafael Petito (FIOCRUZ, Brazil)

13h00-15h00 lunch/free time

14h15-16h 00 Course 3D Model for Cell Culture

Vanessa M Freitas (USP, Brazil)

Aplicações das matrizes extracelulares: Matrigel™ e o novo PuraMatrix™ para Cultura 3D

Aline Fukuzawa - Especialista de Produto BD Biosciences

16h00- 17h30 Symposium 6: ECM in chronic/degenerative diseases

Chairperson: Vilma Martins

16h00- 16h45 Heparin Cofactor II Modulates the Response to Arterial Injury

Douglas Tollefsen (Washington Univ, USA)

16h45-17h15 Prion protein binding to laminin: neurotrophic properties to be explored in degenerative diseases

Vilma Martins (Inst. Ludwig, Brazil)

17h15-17h30 Selected abstract: Glycosaminoglycan anticoagulant properties from atherosclerotic lesion

Lisandra A C Teixeira (UFRJ, Brazil)

17h30- 18h30 Conference

Chairperson: Estela Bevilacqua

Biomechanical and signaling roles of extracellular matrix in regulating early morphogenesis

Raymond Keller (Univ. Virginia, USA)

18h30- 19h00 coffee break

19h00- 21h00

Poster Session II

November 4th

8h30 Symposium 7: ECM and stem cell biology and therapy

Chairperson: Tatiana Coelho-Sampaio

08h30- 09h00 Laminin and stem cell therapy to treat spinal cord injury

Tatiana Coelho-Sampaio (UFRJ, Brazil)

09h00- 09h30 The use of xeno-free, defined extracellular matrices in the derivation of human embryonic stem cells for clinical applications

Adriana Bos-Mikich (UFRGS, Brazil)

09h30- 09h45 Selected abstract: Effect of dermatan sulfate in the migration and proliferation of endothelial progenitor cells after arterial injury in mice

Juliana A P Godoy (UNICAMP, Brazil)

09h45-10h00 Selected abstract: Quail neural crest cell differentiation: the effects of FGF2 and microenvironment

Denise A Bittencourt (UFSC, Brazil)

10h00- 10h30 coffee break

10h30-11h30 Closing Conference

Chairperson: Marimélia Porcionatto

Multivectorial model for thymocyte migration: a Voyage from physiology to pathology

Wilson Savino (FIOCRUZ, Brazil)

Closing Ceremony

ABSTRACTS

C1. The Metastatic Niche: Adapting the Foreign Soil

David Lyden (Cornell University, USA), dcl2001@med.cornell.edu

C2. Cell-Cell Interactions Defines the Metastatic Microenvironment of Tumor Cells

Lubor Borsig

Institute of Physiology, University of Zürich, Switzerland. lborsig@access

Abstract: Recent evidence strongly suggests that cancer progression is dependent on the microenvironment consisting of stromal and inflammatory cells. Interactions of tumor cells with endothelium in a microvasculature of distant organs determine the outcome of metastasis. Here we provide evidence for the molecular mechanism involved in the tumor cell-mediated activation of endothelial cells leading to formation of a metastatic niche. Selectin-mediated cell-cell interactions of tumor cells with platelets and leukocytes induce endothelial production of the inflammatory chemokine, which is the key chemoattractant for monocytic cells. Inhibition of monocyte recruitment strongly reduced survival of tumor cell and metastasis. Similarly, L-selectin deficiency reduced the recruitment of monocytic cells, and attenuated metastasis. Our findings demonstrate that the selectin-dependent endothelial expression of chemokines contributes to the formation of a permissive metastatic microenvironment.

C3. Biomechanical and Signaling Roles of Extracellular Matrix in Regulating Early Morphogenesis

*Ray Keller¹, Lance Davidson², Douglas DeSimone³, Bette Dzamba³, Toshiyasu Goto⁴,
and Paul Skoglund¹*

University of Virginia, Departments of Biology¹ and Cell Biology³, University of Pittsburgh, Department of Engineering², USA, ICORP Organ Regeneration Project, Japan Science and Technology Agency, Tokyo, Japan⁴ rekek@virginia.edu

Live imaging with time-lapse confocal microscopy reveals the dynamics of the assembly and remodeling of fibrillar fibronectin matrix at tissue interfaces during early *Xenopus* (frog) development and suggests both mechanical and signaling roles for this matrix. Disrupting the non-canonical Wnt/planar cell polarity (PCP) pathway reveals a role for this pathway in normal assembly of fibronectin at planar tissue interfaces, and a second role in the mediolateral polarization of cell protrusive activity (mediolateral intercalation behavior: MIB) that is essential for the convergent extension movements of gastrulation. Blocking integrin $\alpha 5 \beta 1$ binding to fibronectin increases the frequency and randomizes the orientation of protrusive activity, suggesting that fibronectin-integrin signaling normally functions in polarizing protrusive activity by suppressing background, random activity. Fibrillin, the extracellular matrix molecule implicated in the human diseases Marfan syndrome and congenital contractural arachnodactyly, is expressed in the dorsal mesoderm of *Xenopus*, and disruption of the fibrillin matrix by three methods results in failure of MIB. Regionally targeted disruptions of the fibrillin matrix result in failure of the mechanical integrity of the notochord and notochordal-somitic boundary. Ectopic expression of fibrillin sub-domains on

the ventral side of the gastrula results in second, dorsal axes, suggesting that fibrillin may have organizer activity.

C4. Signaling Pathways Involved in the Effect of Heparin on Endothelial Cells

H.B. Nader, hbnader.bioq@epm.br

Disciplina de Biologia Molecular, Departamento de Bioquímica, UNIFESP, São Paulo, São Paulo, SP, 04044-020

Heparins (Hep), low molecular weight heparins (LMWH) and sulfated fucans (SF) from brown seaweed are antithrombotic drugs that specifically up-regulate the synthesis of an antithrombotic heparan sulfate (HS) when exposed to endothelial cells. The effect is specific for endothelial cells. The binding is fast and saturable with an apparent K_D of 83 nM to adherent cells and 44 nM to the extracellular matrix (ECM) in the absence of cells. By confocal and electron microscopy, the compounds bound only to the ECM, co-localizing with fibronectin. This was confirmed by flow cytometry using syndecan-4 monoclonal antibody as well as wheat germ agglutinin that label cell surface; on the other hand using both Hep and SF as well as a fibronectin polyclonal antibody no binding was observed. Furthermore, the stimulus in HS synthesis is not elicited by the drugs in the absence of ECM. When the cells were exposed to the compounds at 37°C, they were internalized and with time localized in lysosomes. However, endocytosis of the drugs was not required for the stimulation of HS synthesis. The effect is not dependent of lysosomal enzymes activity, since when the cells were incubated with chloroquine, a lysosomotropic amine that raises the lysosomal pH thus inhibiting enzymatic degradation of internalized compounds, the stimulus on HS synthesis was still observed. Signal transduction pathways were then investigated. Endothelial cells stimulated by these antithrombotic drugs showed activation of phosphotyrosine kinases. Both western blotting and confocal microscopy analyses revealed activation of the focal adhesion complex proteins as FAK, Src and paxillin as well the MAP kinase protein like ERK 1/2. The involvement of protein tyrosine kinases and Ras-MAP kinase pathway was confirmed by the use of specific inhibitors as well as ras-transfected endothelial cells. Other experiments clearly demonstrated the role of phospholipase C- β , intracellular Ca^{+2} mobilization and the nitric oxide pathways the synthesis of HS. To our knowledge this is the first study clearly showing the cell signaling pathways related to the effect of heparin and heparinomimetics on endothelial cells. *Supported by Fapesp, CNPq, CAPES, FINEP.*

Course: 3D Model for Cell Culture

Vanessa M Freitas (USP, Brazil), vmfreitas@usp.br

Cell culture provides a defined assay to study cell biology and pathology *in vitro*. Two-dimensional culture has been the paradigm for a typical cell culture system. However, the seminal work of Bissell and coworkers demonstrated that human breast epithelial cells recapitulate glandular phenotype when cultured in 3D analogs of their native

microenvironment. As a result, biologists and bioengineers have investigated different three-dimensional scaffolds that recapitulate aspects of the native cellular microenvironment for in vitro cell culture. Basement membranes serve as key components in 3D culture. These thin continuous sheets of specialized extracellular matrix separate epithelial and endothelial cells from underlying stroma and play important roles in cellular homeostasis and tissue architecture. This course will introduce the properties of three-dimensional cell culture, main substrates and biological scaffolds used. Specific methodologies will focus on both qualitative and quantitative analysis of three-dimensional cell culture.

S1.1. Regulation of Trophoblast Cell Behavior by Extracellular Matrix

*Ann E. Sutherland, Erin K. Klaffky, Isabel M. Gonzalez, and Bradley Phillips
as9n@virginia.edu*

Department of Cell Biology, University of Virginia Health System, Charlottesville, Virginia, USA

Extracellular matrix has specific effects on cell behavior, which affect many aspects of early development. In the early postimplantation mouse embryo the laminin isoforms laminin-111 and laminin-521 have distinct effects on trophoblast cell behavior, which influence the process of implantation. Laminin-111 promotes migration and decreases spreading and proliferation, while laminin-521 decreases cell movement and promote spreading and proliferation. When presented as adjacent substrates however, laminin 1 acts as a non-permissive substrate for migration and invasion, causing cells to form a boundary at the junction between substrates lacking laminin-111 and those containing any laminin-111. Laminin-111 also affects cell-cell adhesion through changes in the localization of vascular endothelial (VE) cadherin. Ectoplacental cone explants become single cells or very small groups on laminin-111 and VE-cadherin is lost from regions of cell-cell contact. In contrast, trophoblast cells maintain strong cell-cell contacts on substrates of laminin-521, and VE-cadherin is maintained in all regions of cell-cell contact. Embryonic laminin-111 is essential for formation of Reichert's membrane, while uterine laminin-521 is required for trophoblast invasion and proper morphogenesis of the yolk sac placenta. Trophoblast cells interact with laminin-111 using integrin $\alpha 7 \beta 1$, whereas they bind to laminin-521 using both integrins $\alpha 7 \beta 1$ and $\alpha V \beta 3$. On laminin-521 these two receptors mediate different behavior; $\alpha 7 \beta 1$ promotes migration while $\alpha V \beta 3$ promotes spreading. These results show that laminin isoforms influence the direction and quality of invasion of trophoblast cells during implantation, and provide epigenetic cues that drive the morphogenesis of the yolk sac placenta.

S1.2. Extracellular Matrix Remodeling at the Maternal-Fetal Interface

Telma M.T. Zorn (USP, Brazil), temtzorn@usp.br

In mammals, pregnancy leads to extensive adaptations of the uterine microenvironment to receive and implant the embryo. Uterine adaptation includes the formation of the decidua

and consequent remodeling of the endometrial extracellular matrix (ECM) components. This is specially so in the mouse uterus where remarkable modifications of fibrillar and non fibrillar ECM occur, particularly in the periimplantation period. Very thick collagen fibrils appear around mouse decidual cells as soon as decidualization is triggered. Incorporation of tritiated proline by decidual cells and posterior radiolabeled detection of thick fibrils with irregular profile have been observed. However, there are evidences that, at least in part, fibril thickening results from the lateral aggregation of previously existent thin collagen fibrils. It is remarkable that collagen fibrils of the interimplantation sites and those of the nondecidualized stroma of the implantation sites remain unmodified. These thick fibrils are composed by, at least, collagen types I, III and V. In addition, an unusual homotrimeric form of collagen type V was identified in the decidualized endometrium of implantation sites but not in the nondecidualized stroma of the interimplantation sites. In mice, besides being degraded by metalloproteinases, collagen fibrils are also phagocitosed by both fibroblasts and decidual cells. At the maternal-fetal interface, collagen type III is the predominant type of collagen suggesting this collagen type favor the embryo-maternal interactions. Besides collagen, adhesives glycoproteins, such as fibrillin 1 and particularly glycosaminoglycans and proteoglycans, are deeply affected by embryo implantation and decidualization. Hyaluronan, which is present in the whole endometrium before decidualization, nearly disappears from the decidualized estroma. In parallel, the aggregating proteoglycan versican increases in the decidualized areas. Moreover, the SLRPs decorin and biglycan also show a differential expression in the endometrium during pregnancy. Before embryo implantation, decorin is present in the endometrium, disappearing as soon as the decidua is formed. Contrarily, biglycan is highly expressed in the decidualized areas. Experimental studies demonstrated the important role of the ovarian steroids hormones on ECM remodeling. All these results together indicate a key role of ECM at the maternal fetal-interface.

S1.3. Hormone-Regulated Expression and Distribution of Versican in Mouse Uterine Tissues

Renato de Mayrinck Salgado (USP, Brazil), bioff@uol.com.br

Remodeling of the extracellular matrix is one of the most striking features observed in the uterus during the estrous cycle and after hormone replacement. Versican (VER) is a hyaluronan-binding proteoglycan that undergoes RNA alternative splicing, generating four distinct isoforms. This study analyzed the synthesis and distribution of VER in mouse uterine tissues during the estrous cycle, in ovariectomized (OVX) animals and after 17 β -estradiol (E2) and medroxyprogesterone (MPA) treatments, either alone or in combination. Uteri from mice in all phases of the estrous cycle, and animals subjected to ovariectomy and hormone replacement were collected for immunoperoxidase staining for versican, as well as PCR and quantitative Real Time PCR. In diestrus and proestrus, VER was exclusively expressed in the endometrial stroma. In estrus and metaestrus, VER was present in both endometrial stroma and myometrium. In OVX mice, VER immunoreaction was abolished in all uterine tissues. VER expression was restored by E2, MPA and E2+MPA treatments. Real Time PCR analysis showed that VER expression increases considerably in the MPA-treated group. Analysis of mRNA identified isoforms V0, V1 and V3 in the mouse uterus. These results show that the

expression of versican in uterine tissues is modulated by ovarian steroid hormones, in a tissue-specific manner. VER is induced in the myometrium exclusively by E2, whereas MPA induces VER deposition only in the endometrial stroma. The intricate modulation of this proteoglycan by ovarian sex hormones strongly suggests the relevance of the ECM to promote a receptive uterine microenvironment for embryo implantation and development.

Selected abstract: Expression of decorin and biglycan in human normal term placenta and in invasiveness- changed trophoblast pathologies

Alexandre Urban Borbely (USP, Brazil) See Poster Session Abstracts pg 23

S2.1. The Extracellular Matrix as a Modulator of Tumor Invasion and Metastasis

William G. Stetler-Stevenson, MD, PhD

Senior Biological Research Service Investigator, NIH sstevenw@mail.nih.gov

The critical role of the tumor microenvironment and the inefficiency of the metastatic process implies that organ specific homeostatic mechanisms, that maintain normal tissue integrity, are robust and inimical to invasive tumor cells. This concept was elegantly demonstrated by Mintz and Illmensee who showed that injection of malignant murine teratocarcinoma cells into a mouse blastocysts resulted in normal, non-malignant tissue formation in genetically mosaic mice. Recently, Hendrix and colleagues demonstrated that a human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of malignant melanoma. Thus the microenvironment of normal tissues possesses conserved barriers that must be overcome during formation of the tumor microenvironment at the primary tumor site, during tumor cell invasion, as well as in the subsequent establishment of metastatic foci. Formation of a tumor microenvironment that facilitates tumor progression is complex and requires the removal of intrinsic physical, biological and chemical barriers. This process is initiated by proteolytic remodeling of the ECM and in particular the basement membrane. These barriers have been described as selective pressures for the metastatic phenotype. It is now well recognized that both the malignant tumor cells as well as a variety of host responses, such as angiogenesis and inflammation, are responsible for the evolution of the tumor microenvironment. Furthermore, specific genes play dual roles in that they both antagonize and promote tumor progression. TIMP-2 has multiple roles in the extracellular matrix, in this presentation we will identify, and describe the mechanisms and inter-relationships of the multiple functions of TIMPs in tissue homeostasis, and the tumor microenvironment.

S2.2. ECM and Soluble Proteins Control Neuronal Cell Migration

Cecilia Hedin Pereira (UFRJ, Brazil), hedin@biof.ufrj.br

Selected abstract: April modulates in vitro thymocyte migration and cell adhesion by interacting with HSPG

Cecilia Rocha (FIOCRUZ, Brazil) See Poster Session Abstracts pg 16

Selected abstract: The role of laminin and CXCL12 in T-cell migration during cardiac allogeneic graft rejection

Ariany Oliveira Santos (FIOCRUZ, Brazil) See Poster Session Abstracts pg 17

S3.1. Laminin Peptides Influencing Carcinoma Behavior

Ruy Jaeger Department of Cell and Developmental Biology, ICB-USP, São Paulo, Brazil
rjaeger@usp.br

Extracellular matrix (ECM), a three-dimensional network of macromolecules including collagens, laminins, fibronectin, nidogen, and proteoglycans, not only represent a solid support to cells, but also contains cryptic domains, which are exposed by proteolysis and elicit biological responses distinct from intact molecules. Cryptic sites are fragments and bioactive peptides, probably present in all ECM molecules. Laminin present multiple cryptic sites related to adhesion, migration, differentiation, angiogenesis and protease secretion. Our results demonstrated that laminin is cleaved in human carcinoma, generating cryptic sites with relevant biological roles (poster by Pinheiro JJV *et al*). This prompted us to study effects of laminin-derived peptides in cancer biology. Our initial findings were related to salivary cancer. Adenoid cystic carcinoma, a salivary cancer with recurrence and metastasis, expresses prominent basement membrane. We showed that basement membrane of adenoid cystic carcinoma is 40-fold thicker compared to basement membrane at dermal-epidermal junction. It is obvious from the basement membrane thickness that an overproduction of laminin occurs *in vivo* in adenoid cystic carcinoma cells. We then decided to study role played by laminin peptides in cells (CAC2) from human adenoid cystic carcinoma. Laminin peptides were SIKVAV, YIGSR, AG73, and C16. Peptides regulate morphology, migration, invasion and protease activity of CAC2 cells. We have also unveiled molecular mechanisms underlying regulatory activities of these peptides. SIKVAV interacts with integrins alpha3beta1 and alpha6beta1, and increases protease activity of CAC2 cells through ERK pathway. Signal generated by AG73 is transduced by syndecan-1 and beta1 integrin, regulating adhesion and protease activity of CAC2 cells. Effects of peptide C16 are similar to AG73. Peptide YIGSR and its receptor 67 kDa combine forces to modulate migratory activities of CAC2 cells. We are currently extending our observations, by studying effects of laminin peptides in different malignant tumors. AG73 regulates invasive biology of human oral squamous cell carcinoma cells (poster by Siqueira AS *et al*), and C16 regulates genes involved in malignant phenotype of breast cells (poster by Santos E *et al*). Our results strongly suggest that laminin peptides are expressed *in vivo* and influence carcinoma behavior. Support: FAPESP (06/57079-4, 08/57103-8), CNPq (470622/2007-5, 304868/2006-0)

S3.2. Platelet Activation as a Novel Target for Galectin-1-Glycan Interactions: Implications in Inflammation, Thrombosis and Metastasis

Gabriel Rabinovich¹, Mirta Schattner² mschattner@hematologia.anm.edu.ar

¹ Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina. ² Laboratorio de Trombosis I, Academia Nacional de Medicina, Buenos Aires, Argentina

Platelets are anucleated blood cells derived from megakaryocytes that are essential for proper hemostasis and thrombosis and also play critical roles in inflammatory processes, tumor metastasis and host defense. When platelets perceive activating signals through their cell surface receptors, they undergo dramatic structural and chemical changes, involving a complex interplay of cell adhesion and signaling molecules. Activated platelets rapidly bind circulating platelets, via membrane glycoprotein complex $\alpha\text{IIb}\beta_3$ and fibrinogen, to form a thrombus or a plug for preventing bleeding at sites of vascular injury. However, platelet aggregation can also occlude atherosclerotic arteries causing cardiac and cerebrovascular diseases. Galectins, an evolutionarily conserved family of animal lectins, have recently emerged as novel regulators of immune cell homeostasis, inflammation and cancer. Extracellularly, galectins can bind to N-Acetylglucosamine-containing glycoconjugates present on the cell surface and extracellular matrix and trigger a cascade of transmembrane signaling events leading to apoptosis, cytokine secretion, cell adhesion and migration. In addition, galectins are engaged in intracellular processes that are essential for basic cellular functions, such as pre-mRNA splicing, regulation of cell growth and cell cycle progression. Galectin-1 (Gal-1), a 14.5 kDa member of this family, preferentially recognizes multiple Gal β 1,4GlcNAc (LacNAc) units, which may be presented on the branches of N- or O-linked glycans on cell surface glycoproteins. We have recently characterized Gal-1 as new mediator of platelet activation. Gal-1 binds to human platelets in a carbohydrate-dependent manner and trigger *outside-in* (platelet spreading) and *inside-out* (expression of neoepitopes in $\alpha\text{IIb}\beta_{\text{III}}$ complex as well as fibrinogen binding) signaling. It also promotes platelet aggregation, secretion of dense and alpha granule content as well as P-selectin and shedding of microvesicles. In addition, Gal-1 favors the generation of leukocyte-platelet aggregates. A further mechanistic analysis revealed the involvement of Ca^{2+} , cyclic nucleotide, MAPKinase and IP3/AKT pathways in Gal-1-mediated control of platelet activation. Moreover, endogenous Gal-1 appears to be a mediator of platelet aggregation induced by classical platelet agonists. In conclusion, the control of platelet physiology by Gal-1 is a novel unrecognized role for this lectin with potential implications at the crossroad of thrombosis, inflammation and cancer.

Selected abstract: RECK-mediated inhibition of glioma migration and invasion

Renato Ramos Massaro (USP, Brazil) See Poster Session Abstracts pg 35

Selected abstract: The RECK B isoform is downregulated by simvastatin in human melanoma cells

Fernanda Augusta de Lima Barbosa (UFPR, Brazil) See Poster Session Abstracts pg 34

S4.1. Defining the Heparan Sulfate Code that Increases FGFR Function During Branching Morphogenesis

Matthew Hoffman

Laboratory of Cell and Developmental Biology, NIDCR, NIH, DHHS, Bethesda, MD,
USA mhoffman@mail.nih.gov

Heparan sulfate (HS) mediates high affinity binding of FGFs to their receptors and FGF10/FGFR2b signaling is critical for the development of multiple organs, including the salivary glands. The developmental activities of FGFs are controlled by a number of factors: 1. The gradients that they form by binding HS in the extracellular matrix (ECM), and 2. The size and sulfate modifications present on the HS that bind to both the FGF and the FGFR. We hypothesize that the different binding affinities of FGFs for HS impacts the strength/duration of FGF signaling and the localization of FGF within the ECM. Reducing HS binding affinity of FGF10 by point mutation in the HS binding pocket converted it into a functional mimic of FGF7. In contrast, reducing the affinity of FGF10 for its receptor, did not alter its HS-binding properties, but affected the extent of the response. These studies provide a framework to explain how HS regulates diffusion and gradient formation of HS-binding FGFs through the ECM. We also hypothesize that the location of FGF10-induced proliferation is determined by an epithelial heparan sulfate proteoglycan (HSPG) with specific patterns of sulfation. The 3-O-sulfotransferases (3-OST), which are the largest family of sulfotransferases involved in HS biosynthesis, form the most rare HS modifications. 3-O-sulfated HS binds to antithrombin and the herpes simplex virus. We have identified a novel role for 3-O-sulfated HS, which binds FGFR2b and increases FGF10-dependent epithelial proliferation and morphogenesis. To study the function of 3-O-sulfation, under-3-O-sulfated kidney HS was modified with 3-OST enzymes. The 3-O-sulfated HS increases epithelial proliferation and branching morphogenesis. There was increased FGF10 signaling and cellular differentiation. Reducing Hs3st expression decreased morphogenesis and was rescued by exogenous 3-O-sulfated HS. Further, 3-O-sulfated HS increased the amount of FGF10 bound to FGFR2b in pull-down assays, suggesting it stabilizes the FGF10-FGFR2b complex. Thus, 3-O-sulfated HS localized at the end buds increases FGFR2b signaling resulting in end bud proliferation, differentiation, and branching morphogenesis.

S4.2. Sulfated Polysaccharides Ensures a Carbohydrate-Based Mechanism for Species Recognition During Sea Urchin Fertilization

Paulo Mourão (UFRJ, Brazil),

pmourao@hucff.ufrj.br, Laboratório de Tecido Conjuntivo, Hospital Universitário Clementino Fraga Filho and Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Caixa Postal 68041, Brazil

The evolution of barriers to inter-specific hybridization is a crucial step in the fertilization of free spawning marine invertebrates. In sea urchins, molecular recognition between sperm and egg ensures species recognition. The jelly surrounding sea urchin eggs is not a simple

accessory structure; it is molecularly complex and intimately involved in gamete recognition. It contains sulfated polysaccharides, sialoglycans and peptides. The sulfated polysaccharides have unique structures, composed of repetitive units of α -L-fucose, β -D-galactose or α -L-galactose, which differ among species in the sulfation pattern and/or the position of the glycosidic linkage. The egg jelly sulfated polysaccharides show species specificity in inducing the sperm acrosome reaction, which is regulated by the structure of the saccharide chain and its sulfation pattern. Other components of the egg jelly do not possess acrosome reaction inducing activity, but sialoglycans act in synergy with the sulfated polysaccharide, potentiating its activity. The system we describe establishes a new view of cell-cell interaction in the sea urchin model system. Here, structural changes in egg jelly polysaccharides modulate cell-cell recognition and species specificity leading to exocytosis of the acrosome. Therefore, sulfated polysaccharides, in addition to their known functions as growth factors, coagulation factors and selectin binding partners, also function in fertilization. The differentiation of these molecules may play a role in sea urchin speciation.

Selected abstract: Structural characterization and anticoagulant properties of a unique heparan sulfate from a mollusk bivalve. *Angélica Maciel Gomes (UFRJ, Brazil)* See Poster Session Abstracts pg 1

Selected abstract: Fucan and the stimulus in the synthesis of antithrombotic endothelial heparan sulfate: relationship between structure and biological activity. *Valquíria P Medeiros (UNIFESP, UFRN, Brazil)* See Poster Session Abstracts pg 3

S5.1. Vascular Extracellular Matrix and Vessel Wall Development

Jessica Wagenseil and Robert P. Mecham, bmecham@cellbiology.wustl.edu

Department of Cell Biology and Physiology, Washington University School of Medicine,
St. Louis, Missouri, USA

An important factor in the transition from an open to a closed circulatory system was a change in vessel wall structure and composition that enabled the large arteries to store and release energy during the cardiac cycle. The component of the arterial wall in vertebrates that accounts for these properties is the elastic fiber network organized by medial smooth muscle. In most animals with a closed circulatory system, pulse pressure and overall blood pressure increase as the cardiovascular system matures during fetal development and early postnatal life. As blood pressure increases during this period, the smooth muscle cells (SMC) change the types and amounts of extracellular matrix (ECM) they produce in order to strengthen the vascular wall and maintain the appropriate mechanical properties. By changing the amount of elastin in the vessel wall through elastin gene deletion or elastin transgene expression, we show that cardiac and vascular development are physiologically coupled, and we provide evidence for a universal elastic modulus that controls the parameters of ECM deposition in the vessel wall. The major changes associated with elastin insufficiency are an increased number of smooth muscle cell layers in the vessel wall and a substantial increase in blood pressure. Interestingly, these animals live a normal life span with no evidence of cardiac hypertrophy. The unique cardiovascular remodeling seen in elastin insufficient mice indicates that the developing vascular cell can adapt its building process to accommodate

environmental changes and produce an altered overall wall structure that operates at different physiologic setpoints.

S5.2. ECM in Experimental Paracoccidiosis Infection

Eva Burger^{1,2}, *Angela Satie Nishikaku*², *Raphael Fagnani Sanchez Molina*²
Departaments: 1DCSBA-UFES; 2IMUNOLOGIA-USP, evburger@usp.br

Paracoccidioidomycosis (PCM) is a systemic mycosis, caused by the fungus *Paracoccidioides brasiliensis* (Pb), that affects healthy individuals living in rural areas in Latin America. There are many clinical forms of the disease, but severe forms are characterized by the presence of numerous disseminated granulomatous lesions, anergy in cellular immunity and high levels of specific antibodies, in contrast, mild forms have with few localized granulomatous lesions, preserved cellular immunity and low levels of specific antibodies. Granuloma formation can be interpreted as a host defense mechanism to destroy or contain this fungus and avoid its dissemination. We used susceptible (S) and resistant (R) mice to Pb infection to study the granulomas and evaluated the effect of drugs that interfere with fibrosis, which causes severe sequelae to PCM patients. We analysed the architecture of the granulomas and associated with the presence of morphologically preserved or destroyed Pb, deposition of some extracellular matrix (ECM) components (collagen fibers types I, II, IV, osteopontin, laminin, biglycan, decorin), presence of relevant cytokines to granuloma formation (γ -IFN, TGF- β , TNF- α) and of matrix metalloproteinases (MMP). We detected all the above mentioned ECM elements and could propose some roles for each one: the thick fibers of collagen type I, more abundant in the R mice may be associated with Pb infection containment; the thin reticular fibers of collagen type III may promote the microenvironment for Pb cell ECM interactions; the marker of newly formed vessels collagen type IV may promote Pb dissemination and favor the influx of inflammatory cells and the proteoglycans biglycan and decorin, both more abundant in R mice may promote fungal containment. The cytokines TNF- α and γ -IFN, this later conspicuously more observed in R mice may promote macrophage activation, enhancing Pb killing by these cells and the control fungal dissemination; TGF- β , more observed in S mice may promote deactivation and inhibition of Pb killing by macrophages, favoring fungal dissemination and osteopontin may favor infection at its onset and promote protection later, because it is more prominent respectively in S and in R mice at these time points. MMP-9 was detected in both S and R mice with active infection. We treated S mice with drugs that interfere with fibrosis. The best indicators of successful local *P. brasiliensis* lysis were the presence of compact granulomas, required to contain *P. brasiliensis*, of a continuous deposit of collagen type 1 arranged in concentric orientation and the production of high concentration of cytokines IL-12 and γ -IFN as well as of NO. Based on these parameters, we can conclude that therapy with γ -IFN and / or Tetracycline seems promising, reducing the fungal load and altering the granulomas architecture to provide *P. brasiliensis* containment without excessive fibrosis. Grants: FAPESP 06-60091-6, 07/56745-3 e CNPq 307492/2006-0

Selected abstract: Laminin α 5 expression after human myoblast transplantation into Rag-/- Gammac-/- mice

Ariany O Santos (FIOCRUZ, Brazil) See Poster Session Abstracts pg 42

Selected abstract: Schwann cells transdifferentiation: possible role of myofibroblast in neural fibrosis during leprosy

Rafael Petito (FIOCRUZ, Brazil) See Poster Session Abstracts pg 45,46

S6.1. Heparin Cofactor II Modulates the Response to Arterial Injury

Douglas Tollefsen, MD, PhD

Dept. of Medicine, Washington University, St. Louis, MO, U.S.A.

dtollefs@dom.wustl.edu

Thrombin is a key participant in blood coagulation, wound healing, and inflammation. Heparin cofactor II (HCII) circulates in the blood and inhibits thrombin in the presence of dermatan sulfate (DS) or heparin. HCII-null mice do not develop spontaneous thrombosis, but they form thrombi more rapidly than wild-type mice after disruption of the carotid arterial endothelium. IV administration of DS prolongs the thrombosis time of wild-type but not of HCII-null mice. Injection of either wild-type recombinant HCII or a variant with low affinity for heparin corrects the abnormally short thrombosis time of HCII-null mice, while a variant with low affinity for DS has no effect. HCII is undetectable in the wall of the uninjured carotid artery. After endothelial disruption, it becomes concentrated in the adventitia, where it binds specifically to DS. These results suggest that HCII interacts with DS in the vessel wall after disruption of the endothelium and that this interaction regulates thrombus formation. Human studies indicate that plasma HCII levels are inversely correlated with in-stent restenosis (neointimal smooth muscle cell accumulation) and atherosclerosis. Atherogenesis is induced in apoE-null mice by feeding them a high-fat diet. After 12 weeks, mice that are also HCII-null have ~64% larger plaque areas in the aortic arch than HCII wild-type mice. Neointima formation is induced by mechanical dilation of the common carotid artery. Three weeks after injury, the neointimal area is 2-3-fold greater in HCII-null than in wild-type mice. DS administered intravenously within 48 h after injury inhibits neointima formation in wild-type but not in HCII-null mice. Thus, HCII deficiency promotes atherogenesis and neointima formation, and treatment with dermatan sulfate reduces neointima formation in an HCII-dependent manner.

S6.2. Prion Protein Binding to Laminin: Neurotrophic Properties to be Explored in Degenerative Diseases

Vilma Martins (Inst. Ludwig, Brazil), vilma@ludwig.org.br

Flavio Beraldo¹, Camila Arantes^{1,2}, Kill Lee¹, Cleiton Machado^{1,4}, Fabiana Caetano⁵, Nicole Queiroz^{1,4}, Marilene Lopes¹, Margareth Magdesian⁶, Tatiana Américo⁷, Rafael Linden⁷, Marco Prado^{5,8} and Vilma Martins¹.

¹ Ludwig Institute for Cancer Research, SP; ² Departamento de Bioquímica, IQ-USP, SP; ⁴ CTP Hospital AC Camargo, SP; ⁵ Programa de Farmacologia Molecular, ICB-UFMG, MG; ⁶ Instituto de Bioquímica Médica, UFRJ, RJ; ⁷ Instituto de Biofísica, CCS, UFRJ, RJ; ⁸ The Robarts Research Institute, Western Ontario, Canadá.

The prion protein (PrP^C) is a highly conserved cell surface glycoprotein expressed in the Central Nervous System. Its conformationally modified isoform, PrP^{Sc}, is responsible for prion diseases. Multiple and diverse functions of PrP^C have been reported which strongly suggests that loss-of-function have a participation in the pathogenesis of prion diseases. PrP^C is a specific ligand for a peptide at the carboxi-terminal of the laminin γ -1 chain (RNIAEIIKDI) promoting neuritogenesis and modulating neuronal plasticity. In fact, this interaction promotes short and long-term memory formation. Using an intracellular Ca²⁺ probe, Fluo- 3 AM, we verified that the γ -1 peptide, in particular its KDI domain, increased intracellular Ca²⁺ in wild type neurons whereas no effect was observed in PrP^C-null neurons. Neuritogenesis as well as Ca²⁺ signaling were abrogated by U73122, a specific inhibitor of the phospholipase C (PLC) and by 2APB, a specific inhibitor of inositol 3-phosphate receptor at the endoplasmatic reticulum. Remarkably, inhibition of a group 1 metabotropic glutamate receptor (mGluR1) with LY367385 decreased the effect of PrP^C- γ -1 interaction upon mobilization of intracellular Ca²⁺, and blocked the activation of PKC. These data are consistent with the hypothesis that PrP^C interacts with extracellular ligands and a variety of transmembrane proteins can be recruited to a multi-component cell surface complex. They also support the idea that PrP^C has neurotrophic properties that can be explored not only in prion disease but also in other neurodegenerative illnesses. Supported by – FAPESP, CNPq, FAPERJ, Institutos do Milenio, FINEP, PrioNet Canada, and HHMI.

Selected abstract: Glycosaminoglycan anticoagulant properties from atherosclerotic lesion

Lisandra A C Teixeira (UFRJ, Brazil) See Poster Session Abstracts pg 2

S7.1. Laminin and Stem Cell Therapy to Treat Spinal Cord Injury

Tatiana Coelho-Sampaio (UFRJ, Brazil), tcsampaio@histo.ufrj.br

Spinal cord injury (SCI) involves a complex pathophysiology, in which the primary injury is followed by a secondary reaction, where inflammation plays a major role. Research efforts to treat SCI have focused on developing strategies to either decelerate secondary damage or rebuilding the nervous tissue, overcoming the presence of growth-inhibitory molecules and the poor regenerative potential of adult central nervous system. Here we report the positive outcomes of treating acute SCI either with a biomimetical polymer of the extracellular matrix protein laminin or with the injection of mesenchymal stem cells isolated from human adipose tissue (hAT-MS). Laminin has been largely implicated in neural development and regeneration in both peripheral and central nervous system, but in spite of that, there were no reports of exogenous laminin contributing to improve the outcome of experimental SCI. We had previously described that laminin underwent fast self-polymerization upon pH acidification, giving rise to a biomimetical polymer, morphologically identical to natural laminin matrices. Local injection of such polymers largely improved motor function after thoracic compression or transection. Polymerized laminin presented both anti-inflammatory and regenerative effects, increasing tissue preservation, reducing macrophage and neutrophil infiltration and the levels of systemic inflammatory markers such as TNF- α , IL-1 β and C reactive protein. Fluorogold-labeled neurons were

detected in the spinal cord, brain stem and motor cortex, indicating re-growth of short and long fibers across transection. In a parallel study we tested the potential of hAT-MSC in improving recovery from cord transection in rats. Human MSC are known to negatively modulate the immune system, which permits cross species studies. Acute local injection led to an increase of 200% of the score registered in the open-field locomotor test BBB at the eighth week after lesion. Such improvement was only observed after 4 weeks, which is compatible with a regenerative effect. This was confirmed by the enhanced expression of the neuronal regeneration marker GAP-43 and of the number of fluorogold-labeled fibers. We propose that acid-polymerized laminin and hAT-MSC are promising therapeutic agents to treat human SCI.

S7.2. The Use of Xeno-Free, Defined Extracellular Matrices in the Derivation of Human Embryonic Stem Cells for Clinical Applications

Adriana Bos-Mikich

Departamento de Ciências Morfológicas, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil., adriana.bosmikich@gmail.com Daniel Collins, Roslin Cells Ltd, Roslin, Scotland UK.

The therapeutic application of most human embryonic stem [hES] cell derivatives has been hindered by the exposure of the lines to culture components of animal or human origin. The vast majority of the existing hES cell lines have been derived and continued to be maintained with mouse or human fibroblasts as feeder layers and matrices which represent a complex mixture or isolated proteins of animal and human origin. Products of animal origin are of concern for infection with recognized or as yet unrecognized infectious agents and diseases such as HIV-1 or HIV-2, hepatitis B or C among others can be transmitted from human donor cells to a recipient in organ or tissues allotransplantations. In this context, it is clear that the derivation and establishment of new human embryonic stem cell lines for therapeutic applications should be performed in defined conditions, ideally in the total absence of components of animal or human origin. Feeder-independent, defined hES cell culture media has been successfully used for the culture of hES cells (Ludwig et al., 2006) for some time. However, the extra-cellular matrix remains a problem as the purified human components of the matrix are expensive to produce and represent a potential source of contamination. More recently, a xeno-free, defined extracellular matrix substitute was launched [CELLStart, Invitrogen™] and proved to be highly effective for the culture of established hES cell lines in a feeders-free system using a defined medium [Stem Pro, Invitrogen™]. One of the areas that needs further research and improvements is the initial derivation process, which seems to depend on unknown components of the medium or of the extracellular matrix and has proven to represent a major challenge for scientists to achieve good repetitive results in a totally defined culture system.

Selected abstract: Effect of dermatan sulfate in the migration and proliferation of endothelial progenitor cells after arterial injury in mice

Juliana A P Godoy (UNICAMP, Brazil) See Poster Session Abstracts pg 40

Selected abstract: Quail neural crest cell differentiation: the effects of FGF2 and microenvironment

Denise A Bittencourt (UFSC, Brazil) See Poster Session

01. BIOCHEMISTRY OF ECM

01.1- Structural Characterization and Anticoagulant Properties of a Unique Heparan Sulfate from a Mollusk Bivalve

Angélica Maciel Gomes, Eliene Oliveira Kozlowski, Vitor Hugo Pomin, Cristiano Lacerda Reis, Mauro Sergio Gonçalves Pavão

1. UFRJ, Universidade Federal do Rio de Janeiro, Av.

Pedro Calmon - Cidade Universitária

Heparin-like glycosaminoglycans are an important subset of complex acidic polysaccharides that represent the third major class of biopolymer. These molecules are very heterogeneous in terms of molecular weight, charge density, physicochemical properties and biological activities. Heparin-like structures appeared very early in metazoan evolution and have been preserved in complex organisms. The heparin is used as an exogenous anticoagulant compound and its effect is due the formation of a ternary complex with antithrombin and the different serine proteases of the coagulation cascade. However, heparin has a large hemorrhagic effect and its use is very restricted. The present study focuses on the structural and anticoagulant characterization of a heparin-like polymer isolated from the tissues of a bivalve mollusk. Interestingly, although the polymer resists heparinases I and III, it is just partially cleaved by nitrous acid, as observed by gel filtration chromatography, where two peaks, denominated P1 (~30-40 KDa, nitrous acid-resistant fraction) and P2. In vivo assays demonstrated that at the dose of 1 mg/Kg, the HS-like glycan was able to inhibit thrombus growth in photochemically injured arteries without any bleeding effect. In the model of venous thrombosis this compound was capable to decrease the thrombus size. In conclusion, our results suggest that this molecule constitute potential therapeutic compound as alternative to heparin.

Keywords: *heparan sulfate, glycosaminoglycans, anticoagulant, antithrombotic*

01.2- Effects of Oversulfated and Fucosylated Chondroitin Sulfates on Coagulation: Challenges for the Study of Anticoagulant Polysaccharides.

Roberto José Castro Fonseca¹, Stephan-Nicollas M.C.G Oliveira¹, Vítor Hugo Pomin¹, André S. Mecawi², Iracema G. Araujo², Paulo A.S. Mourão²

1. UFRJ, Universidade Federal do Rio de Janeiro, Rua Rodolpho Paulo Rocco, 255 Ilha do Fundão, 255 Rio de Janeiro-RJ. 2. UFRRJ, Universidade Federal Rural do Rio de Janeiro, 2Departamento de Ciências Fisiológicas, Instituto de Biologia, BR465, Km 7, Sero

We report the effects of a chemically oversulfated chondroitin sulfate and a naturally fucosylated chondroitin sulfate on the coagulation system. The former has been recently identified as a contaminant of heparin preparations and the latter is a glycosaminoglycan extracted from extracellular matrix of sea cucumber. The mechanism of action of these polymers on coagulation is complex and target different components of the coagulation system. They have serpin-independent anticoagulant activity, which preponderates in plasma. They also have serpin-dependent anticoagulant activity but differ significantly in the target coagulation protease and preferential serpin. Their anticoagulant effects differ even more markedly when tested as inhibitors of coagulation proteases using plasma as a source of serpins. It is possible that the difference is due to the high availability of fucosylated chondroitin sulfate whereas oversulfated chondroitin sulfate has strong unspecific binding to plasma protein and low availability for the binding to serpins. When tested using a venous thrombosis experimental model, oversulfated chondroitin sulfate is less potent as an antithrombotic agent than fucosylated chondroitin sulfate. These highly sulfated chondroitin sulfates activate factor XII in vitro assays, based on kallikrein release. However, only fucosylated chondroitin sulfate induces hypotension when intravenously injected into rats. In conclusion, the complexity of the regulatory mechanisms involved in the action of highly sulfated polysaccharides in coagulation requires their analysis by a combination of in vitro and in vivo assays. Our results are relevant due to the urgent need for new anticoagulant drugs or alternative sources of heparin.

Keywords: *antithrombotic drugs, heparin, sulfated polysaccharides*

01.3- Anticoagulant Activity of aHeparin from Ascidian Phallusia Nigra (Chordata: Tunicata)

Christiane Sobral Silva, Eliene O. Kozlowski, Mauro S. G. Pavão

1. UFRJ, Universidade Federal do Rio de Janeiro, Rua Rodolpho Paulo Rocco 255, Cidade Universitária

Heparin is a well-known glycosaminoglycan for its ability to prevent blood coagulation. Although heparin from mammalian origin is clinically used as an anticoagulant drug, the appearance of bovine prion diseases and the haemorrhagic effect of high doses of heparin have limited its use. Therefore to look for new sources of heparin-like compounds is an important goal of glycobiologists. In this work, a heparin-like glycan was extracted from ascidian *Phallusia nigra* and purified using anion-exchange chromatography on a DEAE-cellulose and MONO-Q/FPLC columns. The ascidian heparin has a low molecular weight glycan (~12 KDa), as determined by PAGE in barbital buffer. The anticoagulant activity was measured by the aPTT assay, in which it showed a ~60-fold lower anticoagulant activity (3 units/mg) than bovine heparin (180 units/mg). Ascidian heparin is able to inhibit factor Xa via antithrombin ($IC_{50} = 3.14 \mu\text{g/mL}$), but thrombin inhibition was very lower ($IC_{50} = 0.74 \mu\text{g/mL}$), as comparing with mammalian heparin ($IC_{50} = 0.143 \mu\text{g/mL}$ and $IC_{50} = 0.90 \text{ ng/mL}$, respectively). Taken together, these results point to a selective activity of the ascidian heparin as a factor Xa inhibitor. A detailed study about the structure of this heparin is being

performed to establish a correlation among the low molecular weight, structural features and anticoagulant activity.

Keywords: *ascidian, anticoagulant, glycosaminoglycan, heparin*

01.4- Age-Related Changes in Glycobiology of Lumbar Intervertebral Discs in Mice

Priscilla Santos, Leonardo Cinelli, Mauro Pavão

1. UFRJ, Universidade Federal do Rio de Janeiro,
Rua Professor Rodolpho Paulo Rocco, 255

Low back pain (LBP) is a serious public health problem, and the main cause of early retirement in many developed countries. Approximately 80% of the world's individuals will suffer, at least once, from a back pain episode, during the lifetime, and a few percentage will evolve to a chronic pathology. Most of the cases involve back lifting muscles and movement dysfunction. The Lumbar Intervertebral Discs (LID) seen to be responsible for 40% of the cases and is the most damaged structure of the condition. The main pathologic feature of the discs is the decrease of glycosaminoglycans (GAGs) concentration and the length of their chains. Studies about models of degeneration are important for the establishment of new therapies or treatment for LBP. In this work, we performed a glycobiology study of LIDs in mice. LIDs from C57 black-6 of 6, 12 and 24 weeks were dissected. The GAGs were extracted and purified. The pure samples were analyzed by agarose and polyacrylamide gel electrophoresis, gel-filtration and ion exchange chromatography. The samples exhibit molecules of chondroitin sulfate and dermatan sulfate. The concentration of GAGs decreases with aging and there are a change in the proportions of the disaccharide units, with decrease of UA-GalNAc (6S) and increase of UA-Gal NAc (2,4 S). The results can show how aging affects the progression of disc degeneration, which could become a good model to study LBP.

Keywords: *Aging, Disc Degeneration, Glycosaminoglycans, Intervertebral Disc*

01.5- Glycosaminoglycan Expression In Co-Cultures of Endothelial Cells and Smooth Muscle Cells. Endothelial Cells Have a Vectorial Secretion Phenotype

*Gabriel Cunha¹, Thaís Ruegger Jarrouge-Bouças¹, Renan Pelluzzi Cavaleiro¹,
Fernando de Souza Fonseca Guimarães², Edvaldo da Silva Trindade², Juliana Luporini
Dreyfuss¹, Helena Bonciani Nader¹*

1. UNIFESP/EPM, Universidade Federal de São Paulo, Rua Três de Maio, 100 São Paulo – SP. 2. UFPR, Universidade Federal do Paraná, Jardim das Américas, Curitiba - PR

It is known that glycosaminoglycans (GAG) play important roles in several cellular processes. Heparan sulfate (HS) could bind and modulate proteins related to hemostasis, and the exposure of endothelial cells (EC) to heparin (hep) stimulates the synthesis of an

antithrombotic HS. Since co-cultures of EC and smooth muscle cells (SMC) are one of the most similar *in vitro* model to represent the vessel wall, co-culture experiments using a permeable polyethylene terephthalate (PET) support that isolates the apical (luminal) and basal compartments were performed to study GAGs expression of co-cultured ECs with SMCs (EC/SMC; SMC/EC). Another goal of this study was to analyze the vectorial secretion of GAGs by ECs cultured alone (EC/-) since a characteristic heparan sulfate proteoglycan (HSPG) of ECs (syndecan-4) is shedded to the culture medium. Scanning electron microscopy (SEM) analysis of EC/SMC co-cultures showed minor alterations in EC and SMC morphology, with preservation of the polygonal shape. PET cultured ECs showed a major adhesion phenotype when compared to ECs cultured in regular glass coverslip. GAG biosynthesis was measured by metabolic labeling with 150 μ Ci [35S]-sodium sulfate/mL for 18h in EC/-, SMC/EC and EC/SMC and SMC/- cultures. It was shown that EC/- cultures have a vectorial GAG secretion pattern, in which over 95% of all secreted GAG is addressed to the luminal environment, and only residual GAG amounts were observed in the basal compartment. In contrast SMC/- showed significant amounts of GAGs secreted to the basal compartment, corroborating the vectorial secretion phenotype observed in EC/- cultures. In the co-culture studies (SMC/EC), the expression of HS present in the cellular fraction by EC was reduced 50%, while the other co-cultures studies showed no significant differences regarding GAGs expression. The treatment of EC cultures with heparin both at the basal compartment and/or at the apical compartment stimulates the synthesis of HS secreted only for apical culture medium. This treatment had no influence in the vectorial GAGs secretion phenotype. These combined results indicate that the GAGs secretion is vectorial in EC cultures, and the heparin treatment suggests the relevance of the GAG secretion mainly to the vessel lumen in the modulation hemostasis.

Keywords: *glycosaminoglycans, heparan sulfate, vectorial secretion, co-culture, vascular biology*

01.6- Glycosaminoglycan Anticoagulant Properties from Atherosclerotic Lesions

*Lisandra Antonia Castro Teixeira, Ana Maria Freire Tovar,
Paulo Antonio de Souza Mourão*

1. UFRJ, Universidade Federal do Rio de Janeiro, Rua Rodolpho Paulo Rocco, 255, 4º andar, sala 4a01, Ilha do Fundão, RJ

In order to investigate if the concentration and/or the anticoagulant activity of arterial wall glycosaminoglycans (GAG) are affected in atherosclerosis we compared normal segments from human aorta (78-year-old woman) with segments exhibiting different grades of atherosclerotic lesions, fibrous plaque (atheroma) or calcified ulcerated lesions. Atheroma and complicated lesions presented significant lower glycosaminoglycan concentrations, 1.52 ± 1.60 (n=15) and 0.97 ± 0.53 (n=18), respectively, when compared with normal segments, 2.41 ± 1.08 μ g hexuronic acid / mg tissue dry weight (n=11). The anticoagulant activity of aortic GAGs was evaluated by a thrombin amidolytic assay using plasma as a source of thrombin inhibitors or purified antithrombin (AT) or heparin cofactor II (HCII). As indicated

by the concentration of GAG necessary to inhibit by 50% the thrombin activity (IC₅₀), no significant difference was observed in the anticoagulant properties of the GAGs from normal and lesionated areas. Thus, the IC₅₀ was 1.40 ± 0.83 , 1.11 ± 0.83 and 1.83 ± 0.86 $\mu\text{g}/\text{mL}$ for normal, atheroma and complicated lesions areas, respectively. Similar results was observed when purified AT or HCII was used instead of plasma. Fractions enriched in heparan sulfate (HS) or condroitin sulfate plus dermatan sulfate (CS+DS) from the three groups were obtained by anion exchange chromatography. All HS fractions exhibit the same anticoagulant properties. Surprisingly, CS+DS fractions from lesionated areas present a higher anticoagulant potential in the presence of AT. This activity was not altered by chondroitinase ABC treatment, indicating the presence of heparin-like chains. Our results show that atherosclerotic lesions present lower GAG content, however this anticoagulant property is not affected.

Keywords: *atherosclerosis, anticoagulant, glycosaminoglycan, heparan sulfate*

01.7- Integument of Adult *Lithobates Catesbeianus*: Glicoconjugates and Glycosaminoglycans Identification and Localization

*Adriana Alves Pelli, Leonardo Paes Cinelli, Paulo Antonio de Souza Mourao,
Lycia de Brito Gitirana*

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Trompowsky s/no

The distribution of specific carbohydrate moieties in the amphibian integument can be relevant due to its functions since the integument has been involved in water and ion movement. Glycosaminoglycans (GAGs) are long unbranched polysaccharides of a repeating disaccharide unit, and are important to water retention and electrolyte control. Lectins as non-immune proteins or glycoproteins are widely used as probes to detect different type glycoconjugates. The main purpose of this work was to identify GAGs and localize glycoconjugates in the adult *Lithobates catesbeianus* integument. Adult males were obtained from a commercial farm in Rio de Janeiro State (ethics committed DAHEICB-021). Integument fragments were processed according to standard histologic techniques. Slices were stained with hematoxylin-eosin (HE), periodic acid-Schiff method (PAS), Alcian blue (AB) staining at pH 1.0 and pH 2.5 and toluidine blue O. For biochemical assay, crude GAGs were applied to Mono-Q FPLC column and after agarose-gel analysis. Previously investigation by thin-layer chromatography (TLC) was employed to drive the lectin choose. Therefore, the sections were incubated with following biotinylated lectins: AAA (fucose), WGA and STL (N-acetyl-glucosamine), PNA (galactose) and Jacalin (acetyl-galactosamine). In the spongy dermis, the mucous glands exhibited AB and PAS positive reaction, while the serous and granular glands showed no reaction to both methods; however, some cells of the small serous glands revealed alcianophilic reaction at apical cytoplasm, suggesting other gland type constituted by mucous and serous cells, being classified as mixed gland. The Eberth-Katschenko (EK) layer that occurs between the spongy dermis and the compact dermis demonstrated weak alcianophilic reaction as well as the hypodermis. Through lectin histochemistry, N-acetyl-glucosamine was observed between keratinocytes of the corneum and spinosum strata as well as in the mucous gland besides the subepidermal connective

tissue and the EK layer. Galactose was identified only in the mucous gland, but the acetyl-galactosamine is visualized in the mucous and small serous gland and in the EK layer. The biochemical results indicated dermatan sulfate and hyaluronan in the integument. This work indicate that some glycoconjugates are specific for epithelial secretory products while others can participate in GAGs structure, important to structural extracellular matrix maintenance.

Keywords: *amphibians, integument, lectin, glycoconjugate, glycosaminoglycans*

01.8- Nanoparticles of Heparins of Marine Invertebrates: Anti-Inflammatory Effect on Model of Inflammatory Bowel Disease.

Celso Luis Ribeiro Belmiro, Caroline Monteiro Fernandes Freire, Morgana Castelo Branco, Heitor Sifert Pereira Souza, Mauro Sérgio Gonçalves Pavão

1. UFRJ, Universidade Federal do Rio de Janeiro, Rua Paulo Rocco 255

Heparin is a polysaccharide of animal origin, mainly used as an anticoagulant in the prevention and treatment of deep vein thrombosis. Recent pre-clinical studies have also shown a potent antiinflammatory effect of this glycosaminoglycan. Despite highly used in clinical practice, heparin has a high-risk of bleeding and its pharmacological effect is only observed after parenteral administration. Therefore, the search for heparin analogues with lower side effects and better oral activity it is extremely important. In this context, several studies have shown promising results. For example, several works indicate the presence in marine invertebrates of heparin-like glycans containing low anticoagulant activity, potent anti-inflammatory effect and no bleeding tendency. It has been shown recently that nanoparticles increase the oral bioavailability of macromolecular drugs, including heparin. The present work describes the effect of nanoparticles of heparin analogues from marine invertebrates in an experimental model of inflammatory bowel disease (IBD) in rats. Preliminary results showed efficient formation of nanoparticles of heparin, with a good range of distribution and size. Performance and efficiency assays of encapsulated heparin, as well as releasing tests in different pH ranges were satisfactory. In IBD, increased production of TNF- α is associated with tissue damage mediated by immune response of the cellular infiltrate. Rectal administration of TNBS induced significant increase in the levels of TNF- α in inflamed colon. The evaluation of the anti-inflammatory action of the heparin nanoparticles in animals revealed a great reduction in cellular infiltrate and other pro-inflammatory parameters, in addition to a drastic reduction in the levels of TNF- α . These results indicate that heparin nanoparticles possess anti-inflammatory effect in an animal model of IBD. Financial support: CNPq, FAPERJ.

Keywords: *Glicosaminoglicanos, Matriz extracelular, Doença inflamatória intestinal, Doença de Crohn*

01.9- Fucan A and tThe Stimulus in the Synthesis of Antithrombotic Endothelial Heparan Sulfate: Relationship Between Structure and Biological Activity

Valquíria P. Medeiros^{1,3}, Edvaldo S. Trindade², Edda L. Leite³, Hugo A. O. Rocha³,
Helena B. Nader¹

1. UNIFESP, Universidade Federal de São Paulo, Rua Três de Maio, 100, 4 andar, Vila Clementino, SP, SP. 2. UFPR, Universidade Federal do Paraná, Rua XV de Novembro, 1299, Centro, Curitiba, PR. 3. UFRN, Universidade Federal do Rio Grande do Norte, Caixa Postal 1524 - Campus Universitário Lagoa Nova, Natal, RN

Fucans is the term used to define a family of α -L-fucose sulfate. The high density of negative charges of fucans may be related to biological activities shown by these polysaccharides. Studies show that fucans of seaweed have pharmacological actions as potential drugs anticoagulant, antithrombotic, antitumor, antiinflammatory, among others. In this work will be investigated the relationship between structure and activity anticoagulant and antithrombotic of the fucan A extracted from marine brown algae *Spatoglossum schröederi*. The fucan A was submitted to carboxirreduction and dessulfatation. The fucan A native (nFucA), carboxirreduced (cFucA), partially (dFucA2h) and totally (dFucA8h) dessulfated were evaluated by chemical and physical-chemical methods. The anticoagulant effect *in vitro* was investigated by the method of USP, and antithrombotic activity *in vivo* by venous thrombosis induced by obstruction of the inferior vena cava in *Wistar* rats. The able to stimulate of the synthesis of antithrombotic heparan sulfate (HS) in rabbit aorta endothelial cells (RAEC) was analyzed in the presence or absence of native or modified fucan A. This same effect was investigated when the cells were exposed to the polysaccharides together with cloroquin or when they were exposed to the fucan at 4°C. The result suggests that the fucan A may stimulate HS synthesis indirectly through their interaction with the extracellular matrix (ECM). To confirm this hypothesis, fucan A was labeled with biotin. This probe was used to quantify the number of sites of fucan A on endothelial cells by an ELISA-like assay by using streptavidin conjugated with europium. By confocal microscopy fucan A was detected bound on the ECM and was co-localized with fibronectin. Our results suggest that the fucan A from *S. schröederi* is a potent antithrombotic agent, which gives more prolonged effect than heparin in a model of venous thrombosis in rats. This effect is evidenced by the ability of fucan A to increase the synthesis of HS by RAEC. As heparin, the effect is mediated by binding of fucan A to ECM of endothelial cells, possibly by interaction as fibronectin and integrin signaling pathway. The results also suggest that sulfate is essential for the biological activity of fucan A since that 50% reduction in the content of sulfate abolishes the effect of cellular and venous thrombosis. CNPq, CAPES e FAPESP

Keywords: Antitrombotic activity, Fucan, Rabbit aorta endothelial cells, Structure conformation of fucans, Extracellular matrix

01.10- Non-Invasive Marker for Detection of Hepatitis C Virus Infection: Increased Serum Hyaluronic Acid in Brazilian Asymptomatic Blood Donors

Itatiana Ferreira Rodart^{1,1}, Madalena M. Pares², Aline Mendes¹, Cleidenice Barbosa Silva², Fabricio O Carvalho², Afonso J P Cortez², Jose Augusto Barreto², Joao Roberto M Martins¹, Helena Bonciani Nader¹

1. UNIFESP, A Federal University of São Paulo, Department of B, R. 3 de maio, numero 100 Vila Clementino. 2. COLSAN/UNIFESP, Blood Collection Benefit Association, São P, Av. Jandira

Background: Infection of Hepatitis C virus (HCV), a public health problem worldwide and can be asymptomatic for years. The main injury caused by HCV is the hepatic fibrosis, as a result of a chronic inflammatory process in the liver characterized by the deposit of components from the extracellular matrix. The gold standard method to evaluate the liver damage associated with HCV is the biopsy and searching for non-invasive marker tests proposed a series of them like platelet count, serum levels of gamma-glutamyl transferase (G-GT), alanine aminotransferase (ALT), hyaluronic acid (HA) among others. Accordingly hepatic fibrosis progression, serum Hyaluronan (HA) level is increasing. Objective: The purpose of this study is to determinate the HCV viral load in asymptomatic infected blood donors and correlate to the content of serum HA, G-GT, ALT and total proteins. Methods: Samples of Brazilian blood donors were screened by two ELISA anti-HCV methods, showed 308 anti-HCV positive samples. HCV-RNA was detected by Nested RT-PCR method followed by HCV genotype characterization by RFLP method; HCV viral load was determined by real-time PCR. Serum determinations of HA, G-GT, ALT and total proteins were performed biochemically; to comparisons, where it was utilized serum determinations of 308 samples of health donors and gender group. Results: Among initial 308 anti-HCV positive samples, they were confirmed 99 HCV-RNA positive samples (32.14%) and most prevalent HCV genotype is 1 (82.83%), followed type 3 (13.13%). The average viral load was $122,371 \pm 35,932$ copies/mL; no difference was showed between the viral load and HCV genotypes. Increase in serum ALT (9.1%), G-GT (41.4%), and proteins content (8.2%) do not correlated positively to the HCV viral load; in contrast, HA serum determinations showed a positive correlation to HCV viral load. HA content was statistically increased in positive RNA-HCV samples (37.2 ± 3.5 ng/mL) than in only positive anti-HCV one (14.7 ± 1.2 ng/mL) and male HCV infected blood donors showed significant increase in HA (34.0 ± 2 ng/mL) serum content than the females (16.5 ± 1.9 ng/mL). Conclusion: In asymptomatic HCV infected blood donors, serum HA content correlates to the presence of HCV, no genotype type observed difference, indicating that HA could be used as non-invasive marker of the HCV infection. The subjects are asymptomatic, but serum levels of HA indicate a potential histological liver alteration.

Keywords: ácido hialurônico, carga viral, G-GT, transaminase, vírus da hepatite C

01.11- The Effect of *Arrabidaea Chica* Extract on the Healing of Partially Transected Achilles Tendon of Rats

Andrea Aparecida De Aro¹, Mary Ann Foglio^{2,1}, João Ernesto De Carvalho^{2,1}, Laurecir Gomes¹, Edson Rosa Pimentel¹

1. UNICAMP, Department of Anatomy, Cell Biology and Physiology-IB, CP6109, CEP13083-863. 2. CPQBA, Chemical, Biological and Agricultural Pluridisciplinary Res., CP6171, CEP13081-970. 3. CPQBA, Chemical, Biological and Agricultural Pluridisciplinary Res., CP6171, CEP13081-970. 4. UNICAMP, Department of Anatomy, Cell Biology and Physiology-IB, CP6109, CEP13083-863. 5. UNICAMP, Department of Anatomy, Cell Biology and Physiology-IB, CP6109, CEP13083-863.

The *Arrabidaea chica* extract has been used for treatment of epithelial lesions in skin and gastric ulceration, however it is unknown how is its effect on the healing of a fibrous tissue like the Achilles tendon. In this work we have analyzed the partially transected Achilles tendons of rats with 60 days. The partial transection was 4 mm from the insertion of the tendon in the calcaneus. Immediately after the transection a concentrated extract of *A. chica* was applied on the wound before the skin suture and during the consecutive 7 days after the transection. The rats were euthanized and their tendons removed for analysis at 7, 14 and 21 days after the injury. Extracellular matrix (ECM) components of the injured region were extracted with 4M guanidinium chloride in 0.05M acetate buffer pH 5.8. The extract was used for protein dosage, SDS-PAGE and western-blotting. For hydroxyproline (HO-Pro) quantification the tissue was hydrolyzed in 6N HCl and then treated with perchloric acid/cloramine T. A higher concentration of HO-Pro (mg/g dried tissue) was found in tendons treated with the plant extract, 7 (50.21 ± 9.88) and 21 (44.77 ± 4.83) days after injury, compared with their respective controls (without *A. chica*), where we found 39.33 ± 3.62 and 34.91 ± 1.95 mg/g dried tissue, respectively. Regard non-collagenous protein concentration, a lower concentration was detected in rats with 7 days after injury and treated with *A. chica*. Western blotting analysis indicated a remarkable presence of type III collagen in the tendon 7 days after transection and treated with the plant extract. No differences were observed at 14 days, but at 21 days more type III collagen was found in injured tendon that received saline in comparison to the transected tendon treated with *A. chica* extract, probably because the repair process has already been completed. Our results suggest the *A. chica* extract has a determinant effect on the synthesis of collagen and also on the repairing process. If the treatment improves the organization of the ECM still will be clarified.

Keywords: *Achilles tendon, Arrabidaea chica, collagen, extracellular matrix, healing*

01.12- Analysis of the MMP-2 and 9 Activities in Achilles Tendon of Rats Submitted to Exhaustive Acute Exercise with Different Resting Periods

Andrea Aparecida De Aro, Danilo Lopes Ferrucci, Frederico Padovan Borges, Dagmar Ruth Stach-Machado, Denise Vaz De Macedo, Edson Rosa Pimentel

1. UNICAMP, State University of Campinas, Institute of Biology, CP 6109, 13083-863, SP-BRAZIL

The main function of tendons is transmitting the force arisen from muscles to the bone. The tendon adapts itself to different mechanical stimulus, through a remodeling process involving metalloproteases (MMPs) and collagen synthesis. Although the mechanisms of these processes are not completely known, previous works have shown that the exercise causes alterations in the collagen content and MMPs activities. However, it is missing a study on the effect of exhaustive acute exercise intercalated with different periods of resting on the collagen content and MMPs activity. Male rats were submitted to 3 daily sessions of exhaustive acute exercise with resting periods of 1 and 3h between the sessions. The animals were euthanized at days 1, 3 and 6. For quantification of HO-Pro the tendons were hydrolyzed in 6N HCl, and treated with perchloric acid/chloramine T, and the absorbance read at 550 nm. For detection of MMP-2 and 9 isoforms activities, gelatin zymography techniques were employed, and the densitometry (pixels x 105) of bands was used to evaluate the alterations in the quantity of the latent (72 kDa) and active (62 kDa) MMPs isoforms. Analysis of our results showed a larger presence of the latent isoform at day 1 ($65,4 \pm 8.4$) and of the active isoform at day 6 (35.2 ± 5.5), both with 3h of resting, in relation to the control (30.9 ± 7.9 and 4.6 ± 1.2 respectively for latent and active isoforms). No differences were detected for MMP-9. As regard the HO-Pro content expressed as mg/g dried tissue, all groups exhibited larger concentration (around $111,82 \pm 10,45$) in relation to the control ($85,48 \pm 10,12$), with 1 and 3h of resting, except at day 3 ($96,83 \pm 13,2$) with 3h of resting. Interestingly at day 6 with 1h of resting, we found more HO-Pro ($137,49 \pm 13,44$) compared with 3h ($118,34 \pm 9,08$), which is according to the smaller presence of the active isoform of MMP-2 found in the 1h resting group. Our results suggest that the 3h pause between the exercise sessions leads to a larger production of the latent and active isoforms of MMP-2, with consequent reduction of collagen.

Key words: *Achilles tendon, acute exercise, collagen, metalloprotease*

01.13- Sulfated Polysaccharides from Marine Sponges (Porifera): An Ancestor Cell-Cell Adhesion Event Based on Carbohydrate-Carbohydrate Interaction

Eduardo Vilanova^{2,1}, Cristiano Coutinho², Guilherme Maia², Radovan Borojevic², Paulo Mourão¹

1. IBqM/HUCFF, Instituto de Bioquímica Médica, Universidade Federal do Rio, Rua Professor Rodolpho Paulo Rocco, no 255. HUCFF, sala 4a01. 2. ICB, Instituto de Ciências Biomédicas, Av. Carlos Chagas Filho, no 373. CCS, bloco F, sala F201

Marine sponges (Porifera) are ancient and simple eumetazoans. They constitute key organisms in the evolution from unicellular to multicellular animals. We now demonstrated that pure sulfated polysaccharides from marine sponges are responsible for species-specific cell-cell interaction in these invertebrates. This conclusion was based on the following observations: 1-) Each species of marine sponge has a single population of sulfated polysaccharide, which differ among the species in their sugar composition and sulfate content; 2-) Sulfated polysaccharides from sponge interact with each other on a species-specific way, as indicated by an affinity chromatography assay. This interaction requires

calcium; 3-) Homologous, but not heterologous sulfated polysaccharide inhibits aggregation of dissociated sponge cells; 4-) We also observed a parallel between synthesis of sulfated polysaccharide and formation of large aggregates of sponge cells, known as primmorphs. Once aggregation reached a plateau, the demand for de novo synthesis of sulfated polysaccharides ceased. Heparin can mimic homologous sulfated polysaccharide on the in vitro interaction and also as an inhibitor of aggregation of the dissociated sponge cells. However, this observation is not relevant for the biology of the sponge, since heparin is not found in the invertebrate. In conclusion, marine sponges display an ancestor event of cell-cell adhesion, based on calcium-dependent carbohydrate-carbohydrate interaction.

Keywords: *aggregation factor, proteoglycans, cell aggregation, primmorphs, heparin*

01.14- Extracellular Matrix Remodeling Is Improved after Treatment with a Synthetic Peptide Derived From Lopap

Linda Christian Carrijo Carvalho, Luana Wlian, Janaina de Souza Ventura, Ana Marisa Chudzinski-Tavassi

1. IBu, Instituto Butantan, Av. Vital Brasil, 1500, CEP: 05503-900

Defective content and remodeling of extracellular matrix (ECM) is a pathological state for some diseases and a hindrance factor for tissue repair. An important event for tissue repairing consists in the synthesis of ECM proteins by fibroblasts in a well-organized and regulated process. This study was designed to investigate the effect of a synthetic peptide, which has been previously characterized as antiapoptotic peptide (AP), on the synthesis of collagen in vivo and its wound healing property in a rat skin lesion-induced model. Preliminary results demonstrated that this peptide was able to induce the synthesis of ECM proteins such as fibronectin, tenascin and collagen in human fibroblast culture. To evaluate this effect in vivo, mice were treated with local injections of AP (i.d.), and the collagen content was quantified in the dermis by histological analysis and Picrosirius red coloration. The area in the dermis which received AP treatment showed a local increase of collagen content in comparison with a non-treated area, which was injected with saline. This effect was observed one week after treatment and sustained for more the three months. In the rat skin wound healing model, AP or saline was applied locally in the lesions, and the wound closure was observed macroscopic and microscopically. The regenerating tissue was subjected to histological analysis, and to the extraction and quantification of collagen, matrix metalloproteinases (MMPs) and glycosaminoglycans. Results obtained showed a slight improvement of wound contraction, a better tissue remodeling and organization of collagen fibers. The collagen content was substantially increased in the AP-treated lesions. On the other hand, the amount of glycosaminoglycans did not change. A significant increase in MMPs was observed only in the third day after the induction of lesions. Zymographic analysis revealed a band of 62kDa, which corresponds to the active form of MMP-2. This band of activity was most intense in the lesions treated with AP. These data altogether demonstrates that AP is able to stimulate fibroblasts and can improve the process of tissue repair in vivo with greater deposition of collagen and better tissue remodeling. Financial support: Fapesp, Finep and CNPq.

Key words: collagen, fibroblasts, Lopap, synthetic peptide, tissue repair

01.15- Unusual Architecture of Collagen V Fiber Related with the Collagen V Alpha-2 Biochemical Profile in Cutaneous Fibroblasts from Systemic Sclerosis

Francine Fontes Ricco Braga¹, Patrícia Martin¹, Ana Paula Pereira Velosa¹, Solange Carrasco¹, Romy Beatriz Christmann de Souza¹, Vera Luiza Capelozzi², Natalino Hajime Yoshinari¹, Walcy Rosolia Teodoro¹

1. FMUSP, Rheumatology Division of the Faculdade de Medicina da USP, Av. Doutor Arnaldo, 455. 2. FMUSP, Department of Pathology of the Faculdade de Medicina da USP, Av. Doutor Arnaldo, 455

Background: The collagen V (COLV) is involved in mutations of collagen diseases, such as systemic sclerosis (SSc), which was observed an accumulation of unusual collagen, also demonstrated trough SSc developed by immunization of collagen V in health rabbits. **Purpose:** Our purpose was to analyze the tridimensional reconstruction (3D) and biochemical profile of COLV alpha-1 and alpha-2 chains in skin fibroblasts culture from patients in SSc. **Patients and Methods:** Skin biopsies of 5 patients according American Collagen of Rheumatology Criteria (mean age: 45.5+11.) and 5 healthy control skin fibroblasts were also obtained from thorax region during mamoplasty (ACR criteria; mean age: 40.6+16.2y). COLV 3D reconstruction was performed in the immunofluorescence confocal microscopy and the COL V expression was analyzed by SDS-PAGE eletrophoresis and immunobloting methods. **Results:** The structure of COL V fiber in 3D reconstruction presented thickness in SSc fibroblasts compared with pattern thin fibers in the healthy controls. We have observed the biochemical profile of the collagen V with the increase of expression of the alpha-2 chain related with controls, besides the alteration of molecular weight of the quoted chain. **Conclusion:** The overexpression and the unusual organization of COLV fibers, besides the biochemical changes, suggest an interference with the fibrillogenesis process in SSc.

Keywords: collagen V, Sistemic Sclerosis, fibroblasts culture, biochemistry

02. MOLECULAR AND CELLULAR BIOLOGY OF ECM

02.1- Senescence Influences on Igf-1, Androgen Receptor, Matrix Metalloproteinase and Dystroglycan Features in the Prostatic Lesions

Amanda Hetzl¹, Wagner Fávaro¹, Fábio Montico¹, Eduardo Cândido¹, Ubirajara Ferreira³, Athanase Billis², Valéria Cagnon¹

1. UNICAMP, Universidade Estadual de Campinas, Departamento de Anatomia/IB. 2. UNICAMP, Universidade Estadual de Campinas, Departamento de Patologia/FCM. 3. UNICAMP, Universidade Estadual de Campinas, Departamento de Urologia/FCM.

Senescence is a determining factor for the occurrence of morphological changes in the prostate, considering hormonal imbalance and paracrine signaling disturbance between the epithelium and the stroma. Thus, the objective of this work was to characterize and associate the α and β -dystroglycans (α -DG, β -DG), androgen receptor (AR), matrix metalloproteinases 2 and 9 (MMP-2, MMP-9) and insulin-like growth factor receptor (IGFR-1) immunolocalization in the peripheral zone of elderly men's prostate showing benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostate cancer (PC). Sixty prostate samples from post mortem patients, aged 60-90 years were divided into 4 groups: Normal; BPH; PIN and PC. The diagnosis of the prostatic lesions was based on architectural disturbance according to the criteria of Mostofi and Price. The results showed intense IGFR-1, MMP-2 and MMP-9 immunoreactivities in the CP and PIN groups than those found in the other groups. The α -DG and β -DG immunolocalization were weaker in the CP and PIN than those found in the BPH and Normal groups. Intense AR immunoreactivity was characterized in the four groups. Thus, it could be concluded that senescence led to epithelial-stromal imbalance, shown by means of decreased α -DG and β -DG levels and increased IGFR-1, MMP-2 and MMP-9 levels, which are primordial elements for the maintenance of glandular paracrine signaling, contributing to the disease progression. Also, these findings indicated that there was direct correlation between IGF-1 and MMPs, pointing towards IGF-1 as a molecule target in the prostate therapy as well as a possible factor for MMPs positive signalization.

Keywords: *Dystroglycans, Matrix Metalloproteinases, Prostate, Senescence, Steroid Hormones*

02.2- Activity of MMP-2 A Related to the Eruption Rate and Periodontal Ligament Resistance in Rodent Incisors Submitted to Hypofunction

Nádia Fayeze Omar¹, José Rosa Gomes^{2,1}, Juliana Santos Neves¹, Eliene Aparecida Orsini Narvaes¹, Pedro Duarte Novaes¹

1. FOP/UNICAMP, Faculdade de odontologia de Piracicaba/UNICAMP, Av. Limeira, 901 Departamento de Biologia Buco-Dental Piracicaba/SP. 2. UEPG, Universidade Estadual de Ponta Grossa, Coronel Cláudio Cavalcante. Campus Uvaranas-Ponta Grossa/PR

In the rodent teeth have been demonstrated that the hypofunction condition increases the rate of tooth eruption. The constant movement that occurs in rat incisor eruption is accompanied by intense activity in periodontal ligament remodeling. The ligament extracellular matrix remodeling occurs by direct action of enzymes known as metalloproteinases (MMPs). In this sense, this study investigated the relationship among: 1) the eruption rate of rat incisors, 2) the periodontal ligament resistance strength and 3) the MMP-2 activity in periodontal ligament. Ten male Wistar rats had their lower left incisors cut, every two days, at the interdental papilla level, using high-drill rotation, after anesthesia by halothane, producing hypofunction condition (HP). Other ten rats were kept in normal condition of eruption (N) and were used as control. The eruption rate was measured with an ocular millimeter from the gingival margin to the top of the tooth in group HP, and even from a mark made in the tooth, previously, in the N. After 1, 7 and 14 days the animals were killed by cervical dislocation under anesthesia, and the tensile strength of the periodontal ligament

was measured using an algometry. After extracting, the periodontal ligament of the animals was scraping with a periodontal curette, collected in microtubes containing culture medium DMEN and used to assess activity of MMP-2. It was applied 0.1 and 0.05µg of samples protein total in zymogram (poliacrilamide 10% gel added with gelatin 5%). In the hypofunctional group (HP) the eruption rate was higher ($p<0.01$) and the resistance strength of the periodontal ligament was lower ($p<0.01$) when compared with normal condition eruption group (N). The MMP-2 activity in hypofunctional group was greater at 1 and 7 days ($p<0.01$). At 14 days there was a tendency for the collagenase activity return to normal. Thus, we conclude that there was a direct relationship between increasing of eruption rate and MMP-2 activity, and decreasing of the force of ligament resistance in hypofunctional group. It may suggest that MMP-2 has a role in the extracellular matrix remodeling process on periodontal ligament of incisor rat.

Keywords: *incisor rat, eruption rate, metalloproteinases, hypofunction*

02.3- Doxycycline Effects on Eruption Rate, Activity and Expression of MMP-2, MT1-MMP And TIMP-2 In Odontogenic Region of the Rat Incisor Tooth in an Accelerated Eruption Condition.

José Rosa Gomes^{1,2}, Nádia Fayez Omar², Juliana dos Santos Neves², Eliene A. Orsini Narvaes², Pedro Duarte Novaes²

1. UEPG, Universidade Estadual de Ponta Grossa, CI Cavalcanti, Campus Uvaranas, Ponta Grossa- PR. 2. UNICAMP, Faculdade de Odontologia de Piracicaba, Av. Limeira, Piracicaba-SP

Doxycycline is an antibiotic that inhibit the metalloproteinases (MMPs) activity in pathological process but its effects on tissues of the rat incisor tooth were not evaluated yet. Thus, doxycycline effects on the eruption, activity and expression of MMP-2, MT1-MMP and TIMP-2 present in odontogenic region of rat incisor submitted to shortened treatment after 14 days were evaluated. Wistar rats were divided in four groups according to eruption condition. Normofuncional (NF), Doxycycline normofuncional (DNF), Hypofuncional (HP) and Doxycycline hypofuncional (DHP). DNF and DHP groups received daily 80mg/w.b. of doxycycline by gavages during 14 days. The lower left incisor teeth of HP and DHP groups were shortened using a diamond high-speed rotating instrument every two days. In NF and DNF groups a reference mark was made with the same instrument. The eruption rates were measured using a calibrated grid under a microscope eyepiece every two days where distances (mm) from the gingival margin to the marks made on the teeth (NF and DNF groups) and up to the end of incisor (HP and DHP groups) were recorded after all rats had been anaesthetized with halothane. After cervical dislocation the hemi-mandibles were removed and immersed in paraformaldehyde 4%. After demineralization in EDTA 4,12% the samples were included in paraplast to obtain sections with 5µm. For zymogram and Western Blot methods a window was open in bone that recover the odontogenic region to collect the tissues which were immersed in DMEN or in an extraction buffer. It was applied 0,2; 0,1 and 0,05µg of samples in zymogram (poliacrilamid 10% gels added with gelatin 5%) and 7µg to Western Blot gel. In each group 3 rats were used to immunohistochemical method, 4 rats

were used to zymogram method and six rats were used to Western Blotting to MT1-MMP. Monoclonal antibodies were used 1:200 (MMP-2), 1:250 (TIMP-2), 1:1000 (MT1-MMP) and 1:100 to Western Blot. Immunohistochemical and immunoblot results were analyzed by Image J software and by ANOVA and Tukey post test, as well as the eruption rate at $P < 0.05$ level. The doxycycline did not change the eruption or either activity or expression of molecules studied however, the hypofunctional condition increased only the eruption rate, the expression of MT1-MMP and TIMP-2. It allows us to conclude that MT1-MMP and TIMP-2 but not MMP-2 present in odontogenic region can be involved in eruption process. Ethics Animal:1083-1 Environment:265/2007 UNICAMP.FAPESP

Keywords: *eruption, incisor, rat, MMPs, doxycycline*

02.4- Features of the Steroid Hormone Receptors on the Ventral Lobe of the Prostate of Rats (Sprague Dawley) During the Senescence and Hormone Replacement

Eduardo Marcelo Cândido, Wagner José Fávaro, Fábio Montico, Amanda Cia Hetzl, Valéria Helena Alves Cagnon

1. UNICAMP, Universidade Estadual de Campinas, Departamento de Anatomia/IB

The senescence is responsible for hormonal imbalance and hormone replacement has been a therapy to restore these steroid levels. Thus, the main objective of this work was to immunolocalize the AR, ERalpha and ERbeta on the prostate ventral lobe in senile rats and to analyze these hormone receptors before and after hormone replacement, associating these results with morphological features. 30 male rats were divided into 6 groups: Senile Control group (SC) received 5 ml/Kg dose of peanut oil subcutaneously; Testosterone group (ST) received 5 mg/Kg dose of testosterone cypionate; Estrogen group (SE) received 0,025 mg/Kg dose of 17beta-estradiol; Castrate group (CA): the rats were castrated by surgery; Castrate-testosterone group (CT): the castrated rats received the same treatment of the testosterone group; Castrated-estrogen group (CE): the castrated rats received the same treatment of the estrogen group. All groups were treated for 20 days and submitted to immunological and morphological analysis. The results showed that there were no significant changes in the prostatic epithelium among the groups: SC, SE and ST. However, hypertrophied elements of extracellular matrix as reticular and collagen fibers were verified in the stroma of the SE and ST groups. Also, inflammatory foci were identified specially in the SE and CE groups. Moreover, intense AR immunoreactivity was characterized on the ventral lobe epithelium from animals of SC and ST groups, moderate in the CT group and weak in the other ones. Moderated AR localization was verified in the stroma of the SC and ST groups and weak in the SE and CT groups. Weak ERalpha immunolocalization was verified in the epithelium of SC, CA and CT groups, moderate in the CE group and absent in the ST group. Weak stromal ERalpha immunolocalization was verified in SC and ST groups, moderate in the CT group and intense in the other ones. Intense ERbeta immunolocalization was characterized in the epithelium of SC group; moderate in ST, SE and CT groups, weak in CA group and absent in the CE one. In the stroma, ERbeta was seen weakly only in the ST group. Thus, the differential distribution of the steroid receptors in both epithelium and stroma compartments

of the prostate as the result of hormone alterations, signaled a specific activity in each one of them, pointing to the essential interactions among these steroid hormone receptors in the maintenance of prostatic homeostasis and the promotion of prostatic diseases.

Keywords: *Immunohistochemistry, Morphology, Prostate, Senescence, Steroid hormones*

02.5- Futher Investigation of Crotamine Uptake Mechanisms

Fernando de Sá Silva¹, Alexandre Pereira¹, Mirian AF Hayashi¹, Gandhi Rádis-Baptista⁴, Eduardo Brandt de Oliveira³, Fábio Dupart Nascimento⁵, Carlos M Maranduba¹, Enrico Jardim Clemente Santos¹, Alexandre Kerkis¹, Irina Kerkis¹

1. Instituto Butantan, Instituto Butantan, Av. Vital Brasil, 1500, Butantan, São Paulo, SP.
2. UMC, Universidade de Mogi das Cruzes, Mogi das Cruze, SP. 3. USP, Universidade de São Paulo, Ribeirão Preto, SP. 4. UFPE, Universidade Federal de Pernambuco, Recife, PE. 5. UNIFESP, Universidade Federal de São Paulo, São Paulo, SP

Introduction: Cell penetrating peptides (CPPs) hold great potential as delivery vectors for use in research and medicine. The current use of these CPPs is limited by insufficient knowledge of their uptake mechanism. Previously, we isolated and characterized crotamine, a rattlesnake venom compound capable to penetrate into the cytoplasm and the nuclei. The interaction of crotamine with centrioles and chromosomes during cell proliferation and division suggests that its uptake depends on the cell cycle. Previously, our group demonstrated that crotamine penetrates into cells via endocytosis and interaction with heparan sulfate. We aim to verify: a) the crotamine effect in cell proliferation; b) its uptake in different cell cycle phases; c) its interaction with different syndecans (1 to 4); and d) clathrin and/or caveolin endocytosis involved on crotamine uptake. **Methods:** For this study, we performed MTT assay, imunofluorescence and flow cytometry using B16-F10, CHO-K1 and mouse peritoneal cells. Crotamine was conjugated with FITC or Cy3 for its visualization into the cell. For the proliferation assay, the crotamine concentration was 0.01-10 μ M. To analyze crotamine kinetic into cell cycle, we synchronized the cells in G0/G1 phases and the uptake was verified each four hours, during 28 hours. To verify the colocalization of crotamine with syndecans and the proteins related to endocytosis, anti-syndecans 1-4, anti-clathrin and caveolin antibodies were used. **Results:** We observed that on the concentration up to 1 μ M, crotamine induced higher cell proliferation (up to 30%). After cell synchronization, we analyzed crotamine fluorescence in different cell cycle phases. This CPP did not reveal any difference in treated cultures, it suggests that crotamine uptake is independent of cell cycle. Crotamine did not present specificity for any syndecans tested, the colocalization of these molecules was observed on the plasmatic membrane and in citoplasmatic vesicles. We also observed crotamine colocalization with clathrin and caveolin. **Conclusion:** Our data indicated that crotamine: a) in low concentration increased cell proliferation; b) uptake did not depend on phase of cell cycle; c) interacted with four syndecans types; and d) can be internalized concomitant by different mechanisms: clathrin and caveolin endocytosis pathways. These data showed that crotamine behaves like others CPPs - Tat and penetratin. Financial support by Fapesp.

Key words: *crotamine, syndecan, endocytosis, celular uptake, proteoglycan*

02.6- Arginine Supplementation Prevents the Angiogenic Damage in Health Tissues Caused by Radiotherapy

Mônica Nunes Prazeres Ribeiro, Flávia Meireles Gombar, Maria Cristina Dornas, Waldemar Silva Costa, Cristiane da Fonte Ramos, Francisco José Barcellos Sampaio

1. UERJ, Universidade do Estado do Rio de Janeiro, AV. 28 de Setembro 87 FUNDOS

Introduction: Radiotherapy is used to treat cancer patients but side effects are an inevitable consequence. One of the organs that use to be affected by pelvic irradiation is the urinary bladder because frequently it is included in the irradiated field of a wide variety of pelvic tumors. Some authors show that diet supplementation is a good and inexpensive option to reduce the radiation side effects. Objective: The goal of this study is to evaluate whether L-arginine supplementation can prevent the long-term bladder complications after radiation. Material and methods: Adult Wistar rats were separated into 3 groups: control group (C) – receiving food and water ad libitum; irradiated group (I) – received a unique pelvic radiation dose of 1164cGy; and irradiated group supplemented with L-arginine for 7 days before and 15 days after the irradiation (0.65 g/kg body weight) – (I+A). All animals were killed 15 days after the irradiation. Hematoxylin and Eosin was used to quantify the vascular density and Weigert Resorcin Fuchsin to evaluate arterial wall thickness. RT-PCR was used to determine the VEGF and FGF genes expression. One way ANOVA followed by Newman-Keuls test was used to analyze the results. Results: The irradiated group presented a significant reduction in the expression of VEGF ($C=0.5\pm0.1$; $I=0.1\pm0.09$; $I+A=0.5\pm0.2$, $p=0.0018$) and FGF genes ($C=1.2\pm0.5$; $I=0.5\pm0.2$; $I+A=0.8\pm0.3$, $p=0.02$), in the vessels number ($C=15.3\pm11.25$; $I=11.19\pm6.3$; $I+A=16.19\pm9.3$, $p=0.0003$) and in the arterial wall thickness ($C=9.71\pm3.9$; $I=6.8\pm3.7$; $I+A=10\pm3.3$, $p<0.0001$). The arginine supplementation prevented all these alterations. Conclusion: The supplementation with L arginine seems to prevent the bladder wall damage in relation to angiogenesis. These results suggest that arginine supplementation could be efficient in protecting health tissues from radiotherapy side effects. Instituição de fomento: CAPES

Key words: *Angiogenesis, Bladder, L Arginine, Radiotherapy*

02.7. Glutamine Supplementation Prevents the Collagen Expression and Extracellular Matrix Damage in Health Tissues Caused by Radiotherapy

Mônica Nunes Prazeres Ribeiro, Beatriz Rodrigues Rocha, Flavia Meireles Gombar, Leilane Maria Barcellos Nepomuceno, Waldemar Silva Costa, Francisco José Barcellos Sampaio, Cristiane da Fonte Ramos

1. UERJ, Universidade do Estado do Rio de Janeiro, Av. 28 de setembro, 87 fundos

Patients who have had pelvic radiotherapy as part of their cancer therapy may experience urinary bladder late effects such as hyperactive bladder, incontinence and dysuria. Some papers show that diet supplementation is a good inexpensive option to reduce the radiation side effects. The nutritional supplementation with L-glutamine amino acid has been shown effective in maintaining the colon wall mucosa structure in irradiated rats. The goal of this study is to evaluate whether glutamine supplementation can prevent the long-term bladder complications after radiation. Adult wistar rats were separated into 3 groups: control group (C) – receiving food and water ad libitum; irradiated group (I) – received a unique pelvic radiation dose of 1164cGy; and irradiated group supplemented with L-glutamine for 7 days before and 15 days after irradiation (0.65 g/kg body weight) – (I+G). All animals were killed 15 days after the irradiation. RT-PCR was used to determine the collagen I and III genes expression. Stereology method was used to evaluate the extracellular matrix and the muscle volumetric density with the Image J software. One way ANOVA followed by Newman-Keuls test was used to analyze the results. The extracellular matrix volumetric density was significantly reduced in the irradiated group, while glutamine supplementation prevented the decrement ($C=36.84\pm4.37$; $I=31.64\pm5.00$; $I+G=35.53\pm2.60$, $p<0.0001$). The expression of collagen I and III genes was also significantly reduced in the irradiated group, while glutamine supplementation prevented the decrement ($C=1.067\pm0.31$; $I=0.579\pm0.17$; $I+G=1.816\pm0.66$, $p=0.0009$; $C=0.99\pm0.28$; $I=0.54\pm0.13$; $I+G=1.07\pm0.28$, $p=0.0080$ respectively). On the other hand, the volumetric density of muscle was significantly reduced in the irradiated group, but glutamine supplementation did not prevent this alteration ($C=36.43\pm6.15$; $I=29.39\pm7.08$; $I+G=31.38\pm3.14$, $p=0.0001$). The supplementation with L glutamine seems to prevent the bladder wall damage in relation to the extracellular matrix volumetric density and collagen expression. These results suggest that glutamine supplementation could be efficient in protecting health tissues from radiotherapy side effects. Instituição de Fomento: CAPES, CNPQ e FAPERJ.

Keywords: *bladder, collagen, glutamine, radiotherapy*

02.8- Estrogen Imprinting Causes Inhibition of Heparanase-1 Expression in the Adult Rat Ventral Prostate

Taize Machado Augusto, Fabiana Kuhne, Hernandes F Carvalho

1. UNICAMP, State University of Campinas, Dept. of Anatomy, Cell Biology,
Physiology and Biophysics, Institute of Biology

Heparanase is an endoglycosidase that degrades heparan sulfate on the cell surface and extracellular matrix. The physiological functions of heparanase include heparan sulfate turnover, embryo development, hair growth, and wound healing. It is implicated in a variety of pathologies, such as tumor growth, angiogenesis, metastasis and inflammation. Heparanase overexpression in a variety of malignant tumors suggests that it could be a target for anti-cancer therapy. Heparanase activity can be regulated by androgens in the rat ventral prostate (VP) (Augusto et al., Cell Tissue Res 332:307, 2008) and by estrogens (Xu et al., Hum Reprod. 22(4):927, 2007) in human endometrium. The promoter region of heparanase gene have estrogen responsive elements (EREs), suggesting that the gene is highly regulated by

estrogens. In this work, we investigated the expression of heparanase-1 in the VP of 90-day-old rat after neonatal exposure to high dose estrogen. Western blotting and RT-PCR revealed a marked reduction in the content of heparanase-1 and its mRNA. Immunohistochemistry showed that heparanase-1 could be found in the stroma but not in the epithelium. To determine whether epithelial absence of heparanase-1 was due to pre- or post-transcriptional regulation we isolated epithelial cells through centrifugation in Percoll gradient and used RT-PCR to investigate the presence of heparanase-1 mRNA. The results showed that epithelial cells did not express heparanase-1. Considering that heparanase-1 transcription is affected by the level of cytosine-methylation in the promoter region, we used the methylation sensitive restriction enzyme HpaII and RT-PCR to show that a single CCGG site at +162 appears methylated in the VP epithelium of estrogenized animals. These results suggest that heparanase-1 expression is blocked in the epithelial cells of the VP by estrogen imprinting by a pre-transcriptional mechanism. In parallel, we are also studying whether the levels of EGR-1 transcription factor, which is known to regulate heparanase-1 expression is also affected by estrogen imprinting. TMA is recipient of a FAPESP DR1 fellowship.

Keywords: *estrogen, prostate, heparanase-1, heparan sulfate*

02.9- Urinary Bladder Collagen Expression is Programmed by Maternal Malnutrition During Lactation In Rat

Jorge Alves, Flávia Gombar, Rafaela Veiga, Fernanda Cavalcante, Francisco José Barcelos Sampaio, Cristiane da Fonte Ramos

1. UERJ, Universidade do Estado do Rio de Janeiro, Av. 28 de Setembro 87 fundos

Some authors have shown that lactation could be a critical period in determining the future endocrine status of the progeny. Previous papers show that the urogenital system can be programmed by maternal malnutrition. However, at this time, the effects of maternal malnutrition on the urinary bladder are unknown. The goal of this study was to evaluate the collagen expression at the urinary bladder of adult animals whose mothers were malnourished during lactation. At parturition, dams were randomly assigned into control group (C), with free access to a standard laboratory diet containing 23% protein; protein-energy-restricted group (PER), with free access to an iso-energy and protein-restricted diet containing 8% protein and energy-restricted group (ER), receiving restricted amount of the 23% protein diet. After weaning, all male pups had free access to the diet containing 23% protein until 90 days old when they were killed. Real Time PCR and immunohistochemistry were used to determine the collagen I expression. Despite a significant reduction in the food consumption and body weight gain in the malnourished groups during the lactation time, at the end of the experiment there was no difference in the body weight ($C=370.8\pm3.5$; $PER=281.7\pm3.3$; $ER=368\pm3.5$) or food intake ($C=318.8\pm59.7$; $PER=281.3\pm40.8$; $ER=276.7\pm114.6$) among the groups. The collagen I expression was significantly decreased when evaluated by Real Time PCR ($C=3.2\pm0.9$; $PER=0.7\pm0.6$; $ER=0.9\pm0.3$; $p<0.03$) and immunohistochemistry. We can surmise that the collagen expression of the urinary bladder was programmed by maternal malnutrition during lactation.

Keywords: *Bladder, Collagen, Q-PCR real time, Malnutrition, Rats*

02.10- Mechanical, Biochemical and Morphometric Analysis of the Femur of MDX Mice

Wilson Romero Nakagaki, José Angelo Camilli

1. UNICAMP, Universidade Estadual de Campinas, IB/DABCFB - Av. Bertrand Russel, s/no - CP 6109 - CEP: 13083-865

Introduction: In *mdx* mice, muscle degeneration starts to become severe in the third week after birth, with the onset of degeneration/regeneration cycles that decrease the emission of mechanical stimuli to bone. Thus, the bone remodeling process provokes the loss of bone mass throughout the growth of the animal. On the basis of previous findings showing an altered metabolism in *mdx* tissue, we tested the hypothesis that the alterations in *mdx* bone might not only be due to muscle weakness, but also to factors inherent to the genetic alterations of the animal. **Objective:** To identify alterations in the femur of 21-day old *mdx* mice, an age when the bone has not yet been under the influence of muscle weakness. **Material and Methods:** Femurs from male animals were used. Thirty animals were divided into two groups: *mdx* and control (C57Bl/10-ScCr). Mechanical testing (3-point bending test), hydroxyproline quantification, thermogravimetric analysis (TGA) and morphometry of the diaphysis were carried out and the results were compared between the two groups. The mechanical properties analyzed were maximum stress (σ_m), yield stress (σ_y) and elastic modulus (E). Diaphyseal cortical bone area and thickness were calculated. The results were analyzed statistically by the Student *t*-test at a level of significance of 5%. **Results:** The mechanical properties were higher in the control group ($\sigma_m = 209 \pm 18$ MPa, $\sigma_y = 136 \pm 16$ MPa, $E = 3114 \pm 404$ MPa) than in *mdx* mice ($\sigma_m = 121 \pm 13$ MPa, $\sigma_y = 94 \pm 9$ MPa, $E = 1891 \pm 862$ MPa). Hydroxyproline content was similar in the two groups (control = 21.05 ± 2.88 mg/g and *mdx* = 21.09 ± 2.52 mg/g). TGA showed that inorganic material weights at temperatures of 600, 700 and 1000°C were always lower in the *mdx* group. Diaphyseal cortical layer area and thickness were two times higher in the control group (0.204 ± 0.02 mm² and 0.065 ± 0.004 mm) compared to the *mdx* group (0.114 ± 0.01 mm² and 0.037 ± 0.005 mm). **Conclusion:** The results demonstrated a lower quantity of inorganic bone matrix and lower mechanical strength of the femoral diaphysis in *mdx* mice compared to the control group. Thus, in *mdx* femurs osteopenia and weakness develop prior to the manifestation of muscle alterations. Supported by CAPES/PROEX and FAPESP.

Key words: *mdx, bone, mechanical test, hydroxyproline, inorganic bone matrix*

02.11- Hyperglycemia and Insulin Replacement Impact in the Rat Prostates MMP-2 and MMP-9 Activities During Pubertal Growth

Sérgio Alexandre Alcantara dos Santos¹, Elaine Manoela Porto², Larissa Mayumi Ribeiro¹, Livia Maria Lacorte¹, Antônio Justulin Junior², Sérgio Luis Felisbino¹

1. UNESP, Universidade Estadual Paulista, Distrito de Rubião Jr., s/nº Botucatu - SP Brasil. 2. Unicamp, Universidade Estadual de Campinas, Rua Monteiro Lobato, 255 - Campinas - SP - Brasi

The matrix metalloproteinases (MMPs) are a family of molecules that are associated with the breakdown of constituents of the extracellular matrix (ECM). Both MMPs and their tissue inhibitors (TIMPs) are involved in the regulation of the ECM metabolism. The most common extracellular pathology in diabetes is the thickening of the basement membrane as a result of the deposition of ECM proteins. The expression and activity of MMPs in diabetes thus far have been reported predominantly in relation to macrovascular and microvascular complications. Here, we investigated if the STZ-induced diabetes interferes with MMP-2 and MMP-9 activities during prostate pubertal growth and the effect of a simultaneous or late insulin replacement. Prepubertal male Wistar rats (40 days-old) were used. Diabetes was induced through administration of a single dose of streptozotocin (STZ; 40mg/kg body weight) at 40-days old. Three (simultaneous) or twenty days (late) after STZ-administration, insulin was replaced (3U/100g) for 17 and 20 days, respectively. Diabetic, diabetic plus insulin (simultaneous and late) and age-matched controls animals were killed by overdoses of pentobarbital after 20 days and 40 days of beginning of the experimental period. Prostatic lobes, ventral, dorsal and anterior, were dissected out, weighted and processed for biochemical analysis of gelatin-zymography. The zymography analysis showed clear bands of MMP-2 and MMP-9 in control, diabetic and diabetic plus insulin replacement. The densitometric analysis of the bands showed that prostatic lobes from diabetic animals presented reduced activities of MMP-2 and -9. Simultaneous and late replacement of insulin restores the MMP-2 and -9 activities even over the control levels. In conclusion, diabetes, directly or indirectly (testicular damage and androgen deprivation), disturbs the structure of the prostate gland growth and led to an effective reduction in the MMPs activities. The insulin replacement, even delayed, restores the glandular structure and the MMPs activities, showing that adverse effects of this disease in the prostate are reversible and MMPs play a role in the glandular structure restoration.

Keywords: *diabetes, extracellular matrix, metalloproteinases, prostate, zymography*

02.12- Could the Association of *Heteropterys Aphrodisiaca* (O.Mach) Infusion and Endurance Exercise Modify the Collagen Content and the Biomechanical Properties of Rat Achilles Tendon?

Juliana Castro Monteiro¹, Marcos de Lucca Moreira Gomes¹, Tatiana Carla Tomiozzo^{1,2}, Mariana Mendes Sbervelheri¹, Wilson Romero Nakagaki¹, Edson Rosa Pimentel¹, Heidi Dolder¹

1. UNICAMP, Universidade Estadual de Campinas, IB, DABCF, CP 6109- Campinas, SP 13083-863. 2. PURO-UFF, Polo Universitário Rio das Ostras - UFF, Rua Recife, s/n, Rio das Ostras - RJ 28890-000

Heteropterys aphrodisiaca, also known as “nó-de-cachorro”, “nó-de-porco” and “cordão-de-São-Francisco”, is used as an aphrodisiac, a tonic or stimulant agent and for the treatment of nervous debility, nervous breakdown and for muscle and bone weakness. Previous studies with this species suggested that the root extract could increase corporal and testicular weight, as well as Leydig cell volume. Since sexual organ weights in animals treated with *H. aphrodisiaca* infusion showed no alterations, it was suggested that this plant could exert an

anabolic effect in rats. The purpose of this study was to evaluate the effect of *H. aphrodisiaca* infusion on tendon properties under endurance exercise. Wistar rats were grouped as follows: CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT-control trained, HT- *H. aphrodisiaca* trained. The training protocol consisted in running on a motorized treadmill, five times a week, with weekly increase in treadmill velocity and duration. Water or *H. aphrodisiaca* (dose: 35 mg/animal/day) was administered daily by gavage for 8 weeks. The rat's Achilles tendons were either frozen for hidroxyproline dosages and biomechanical analysis or preserved in Karnovsky fixative and processed for histomorphological analysis by polarized light microscopy. The hidroxyproline content increased significantly in the HT group. Biomechanical analysis showed significant increase in maximum stress and modulus of elasticity in tendons of the HT animals. The most intense birefringence was observed as in compression as in tension regions of the tendon of HT animals, which may indicate a higher organizational level of the collagen bundles. Therefore, the association of endurance exercise with *H. aphrodisiaca* resulted in stronger and better-prepared tendons to support high loads from intense muscle contraction. This association could avoid the marked degradation of collagen and others proteins occurring during endurance exercise or stimulate tendon remodeling by promoting higher collagen content and probably increased cross-links. Despite the clear anabolic effects of *Heteropterys aphrodisiaca* and the endurance exercise association, no side effects were observed, like those provided by synthetic anabolic androgenic steroids. FAPESP, CAPES/PROEX

Keywords: *Achilles tendon, Biomechanical, collagen, endurance exercise, Heteropterys aphrodisiaca*

02.13- Study of the Interaction between Anastellin and the Tenth Repeat of Fibronectin Type III by Molecular Docking: Insights into Fibronectin Fibrillogenesis

Gabriel Limaverde Soares Costa Sousa^{1,2}, Elaine P. S. Gee^{2,3}, Pedro G. Pascutti¹,
Donald E. Ingber^{2,3}

1. IBCCF-UFRJ, Instituto de Biofísica Carlos Chagas Filho - UFRJ, CCS, Cidade Universitária - UFRJ, Rio de Janeiro/RJ – Brazil. 2. VBP - HMS, Vascular Biology Program - Harvard Medical School, Children's Hospital, Harvard Medical School, Boston/MA – USA.

3. WIBIE, Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge/MA – USA

Fibronectin (FN) is a large glycoprotein composed of several globular domains (type I, II and III) that appears in a soluble dimeric form in plasma that polymerizes into insoluble fibrils when it incorporates into extracellular matrix (ECM). Through a process that is not fully characterized, fibril formation appears to require cell traction forces, which leads to partial unfolding of FN molecules. FN type III modules (FNIII) fold into seven β -strands (named A–G) divided between two β -sheets. Under tension, these strands gradually peel apart from the globular core. FNIII modules, which have been proposed to mediate the fibrillogenesis process, share a structural motif that enables an elastic response to mechanical stress given the absence of intra-module disulfide bonds to stabilize the fold. Anastellin, a

C-terminal fragment from the first type III domain (1FNIII), induces fibril formation in vitro when incubated with soluble FN. The resulting polymer, termed superfibronectin (SFN), resembles the natural form of FN found in ECM, and exhibits enhanced adhesive properties. The process of SFN formation is also unknown, and it is unclear whether the mechanism behind its assembly is similar to the one used during natural matrix formation. Despite evidence that anastellin binds to FNIII modules, the molecular interactions that mediate this interaction remain unknown. In an attempt to better define the interacting interface between FNIII modules that may occur during FN fibrillogenesis, we carried out molecular docking and molecular dynamics simulations (using AUTODOCK and GROMACS software packages) between the anastellin-like folding intermediate of 1FNIII and 10FNIII, since both modules have known unfolding intermediates that were previously suggested to be involved in FN fibrillogenesis. Our simulation results suggests the E strands from each module may bind in an antiparallel manner, which excludes solvent from the previously exposed hydrophobic cores, thereby promoting molecular assembly. If experimentally confirmed, this model may contribute to understanding of a key step in the fibrillogenesis process that is critical for formation of FN fibrils in the context of both SFN formation, and natural ECM assembly. The knowledge generated by these studies may open new perspectives in biotechnology, guiding the development of self-assembling nanobiopolymers and nanofibrils for clinical use.

Keywords: *Anastellin, Fibronectin Fibrillogenesis, Molecular Docking, Self-assembling biopolymers, Superfibronectin*

02.14- Disturbed of Purinergics Receptors Activation in GAG-Deficient CHO-745 Cells

Rafael de Lima¹, Gioconda Emanuella Diniz de Dantas Moura¹, Edgar Julian Paredes Gamero¹, Helena Bonciani Nader¹, Giselle Zenker Justo¹, Ivarne Luiz dos Santos Tersario^{1,2}

1. UNIFESP, Universidade Federal de São Paulo, R. Três de Maio, 100. 4º Andar. 2.

UMC, Universidade de Mogi das Cruzes, Av. Dr Cândido Xavier de Almeida Souza, 200

Proteoglycans and integrins mediate cell attachment to various extracellular ligands. These molecules physically bridge the ECM and cytoskeleton, and act as transducers of “outside-in” and “inside-out” signaling. Due to this, a myriad of cellular functions such as differentiation, proliferation, migration, apoptosis, and inflammatory response can be affected. It has been shown that the purinergic P2X7 receptors binds integrin-laminin complex and its activation not only promote a cytosolic influx of Ca²⁺, but it also results in rearrangement of cytoskeleton. Here, we report on the effects of glycosaminoglycans (GAGs) deficiency on purinergic activation in CHO cells. CHO-K1 and its defective mutant on the biosynthesis of GAGs, CHO-745 cells, were examined for their cellular responses to the purinergics receptor agonists. Differences in proliferation and cell death were found between CHO-K1 and CHO-745 when stimulated by ATP and analogs (BzATP, ADP, UTP, UDP). In addition, intracellular Ca²⁺ response was also altered. We verified that in CHO-745 cells, ATP and BzATP (P2X7 selective agonist) were less potent to mobilize intracellular Ca²⁺. Similar expression at protein level of P2X7 receptor was observed in GAG-deficient CHO-

745 cells and CHO-K1 cells, this result suggested that the differences observed is not associated with its expression. Thus, both CHO lineage cells express the P2X7 receptor and its activation can be altered in GAG-deficient CHO-745. Also, aberrant Ca^{2+} steady-state levels were observed in CHO-745 cells, in non-stimulated CHO cells the basal levels of cytosolic Ca^{2+} in CHO-745 cells was 30% smaller than the basal levels of CHO-K1 cells. The results suggested the involvement of GAGs in the control of purinergic receptor activation in CHO cells.

Keywords: Ca^{2+} , P2X7 receptors, purinergic receptors, CHO cells

02.15- Laminin Expression in the Anuran Integument of Two Bufonids of The *Rhinella* Genus

Flavia Felsemburgh, Pablo Almeida, Lycia de Brito-Gitirana

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Trompowsky s/no

Laminin is a large noncollagenous glycoprotein found in the extracellular matrix, composed of combinations of five known alpha, four beta and three gamma chains in mammals. It represents the major component of basement membrane produced by the majority of epithelial cells, including the endothelial cells. Through specific interactions with integrins, dystroglycan, and other receptors, laminin contribute to cell adhesion, growth, migration, proliferation and differentiation, maintenance of tissue phenotypes and survival, having enormous clinical significance. Since amphibian integument has a large structural complexity that reflects its physiological properties, performing several functions, the amphibian can survives in different environments. In order to increase the knowledge about anuran integument, the purpose of this study was to identify the laminin expression in the integument of two bufonids. Adult males of *Rhinella ornata* collected in São Conrado and *Rhinella icterica* collected in Teresopolis, Rio de Janeiro (license 12164-1 – IBAMA/MMA; ethics committed DAHEICB 017) were used. Integument fragments from dorsal and ventral regions were fixed with formaldehyde and processed according to the paraffin embedding standard histological technique. Five micrometers slices were stained with hematoxylin-eosin (HE), Periodic Acid-Schiff reaction (PAS) and used for immunolocalization of laminin at 1:60 dilution (Sigma, Rabbit Anti-laminin, L9393). The results showed that the laminin occurs just below the epidermis in the basement membrane, which also was evidenced by PAS method, indicating the presence of neutral glycoproteins. The laminin was also visualized surrounding the secretory portion of the mixed and granular glands as well as around of the blood vessels. The laminin expression in anuran integument indicates its participation in the normal structure of the basal lamina, since basal laminae are important sheets of specialized extracellular matrix that underlie and surround groups of cells, such as epithelia or endothelia, enabling the cells to orientate their basal/apical polarity and creating a microenvironment for them. Moreover, the laminin is a component of the hemidesmosome that links the basal keratinocytes to the adjacent connective tissue. Then, the laminin contribute to the maintenance of the anuran integument integrity and its functionality. Support: CAPES, FUJB/UFRJ.

Keywords: basement membrane, integument, laminin, *Rhinella icterica*, *Rhinella ornata*

02.16- Organization of Collagen Bundles During Tendon Healing in Rats Treated with L-Name

Tatiana Carla Tomiosso^{1,2}, Wilson Romero Nakagaki¹, Laurecir Gomes¹, Stephen Hyslop¹, Edson Rosa Pimentel¹

1. UNICAMP, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz s/n CP 6109, 13083-863 Campinas, SP, Brazil. 2. UFF, Universidade Federal Fluminense, Departamento Interdisciplinar- PURO-UFF, Rio das Ostras – RJ- BR

The Achilles tendon can support high tension forces and may experience lesions. The damaged tissue does not regenerate completely; the organization and mechanical properties of the repaired tendon are inferior to those of a healthy tendon. Previous reports have demonstrated that nitric oxide (NO) plays important role in the wound repair, but a few studies have discussed about its effect on the reorganization of ECM components. In this work we examined the structural reorganization and repair in Achilles tendon after injury in rats treated with the NO synthase inhibitor N^o-nitro-L-arginine methyl ester (L-NAME). The right Achilles tendon of male Wistar rats was partially transected. One group of rats was treated with L-NAME (~300 mg/kg/day, given in the drinking water) for four days prior to sectioning of the tendon and throughout the post-operative period. Control rats received water without L-NAME. The tendons were excised 7, 14 and 21 days post-injury and used to quantify hydroxyproline and for mechanical tests. Tendons were also processed for histomorphological analysis by polarized light microscopy. Polarized light microscopy showed that the collagen fibers were disorganized by day 7 in non-treated and L-NAME-treated rats. In non-treated rats, the organization of extracellular matrix was more homogeneous by days 14 and 21, compared to day 7, although this homogeneity was still less than in normal tendon. In contrast, in injured tendons from L-NAME-treated rats, the collagen fibers were still disorganized on day 21. Tendons from L-NAME-treated rats had more hydroxyproline but lower mechanical properties compared to those from non-treated rats. These results indicate that NO modulates tendon healing, with a reduction in NO biosynthesis, delaying reorganization of the extracellular matrix, especially collagen. Supported by Capes

Keywords: *collagen, tendon, biomechanics, nitric oxide, L-NAME*

02.17- Increased Col5alfa1 and Col5alfa2 Chains in Fibroblast Culture from Skin and Lung Oo Systemic Sclerosis (Ssc)

Jymenez de Moraes, Patrícia Martín, Solange Carrasco, Romy Souza, Ana Paula Velosa, Maria Lúcia Katayama, Cláudia Goldeinstein-Schainberg, Natalino Hajime Yoshinari, Walcy Rosolia Teodoro

1. FMUSP, Faculdade de Medicina da Universidade de São Paulo, Avenida Doutor Arnaldo 455 Cerqueira César

Background: Systemic sclerosis (SSc) is a fibrotic disease characterized by an increased extracellular matrix deposition, vasculopathy and autoimmune dysfunction. We have found an excessive and distorted type V collagen expression in fibroblasts from lung and skin in

SSc patients. Purpose: To evaluate the morphological pattern of type V collagen and COL5 α 1 and COL5 α 2 gene expression in fibroblasts of lung and skin from SSc patients. Patients and Methods: Lung biopsies of 7 patients and 3 controls as well as skin specimens of 6 patients and 6 controls were obtained from SSc patients according American Collagen of Rheumatology Criteria (ACR). For fibroblast culture skin and lung evaluation we used semiquantitative score: intense expression (1-4), fibroblast number/field (1-2) and collagen fibers architecture (1-3). The total evaluations were: mild (3-5), moderate (6-7) and severe (8-9). Molecular analyzes COL5 α 1 and COL5 α 2 gene expression in fibroblasts of skin and lung was performed in Real Time PCR-RT. Results: Fibroblasts of lung and skin from SSc patients showed increased thickening and irregular bundles of COL V distributed in parallel and perpendicular arrangements resulting in a dense network pattern. The semiquantitative analyze in the cutaneous fibroblast in SSc and controls showed changes and an increase of COLV fiber structure expression ($82,50 \pm 9,5\%$ vs $47,5 \pm 9,5\%$ $p=0,002$) and in lung fibroblast presented increase and same morphological pattern in COLV fibers ($38,87 \pm 2,99\%$ vs $20,33 \pm 7,50\%$ $p=0,002$). The molecular evaluation demonstrated an increased of COL5 α 1 and COL5 α 2 mRNA expression in SSc fibroblast skin when compared to control ($1,375 \pm 0,373\text{au}$ vs $0,0047 \pm 0,0013\text{au}$, $p=0,05$). Similar results were observed in lung: ($1,61 \pm 0,654$ vs $0,99 \pm 0,51 \text{ au}$; $p=0,05$). The proportion COL5 α 1/COL5 α 2 mRNA in fibroblast lung and skin was higher in SSc patients than in controls being the chains ratio 1:2. Conclusion: Intense expression of abnormal COLV was demonstrated in skin and lung fibroblast culture of SSc patients. This unusual COLV structural and molecular profile can justify the morphological pattern, and maybe, cutaneous thickening as well pulmonary fibrosis viewed in SSc patients. These results provide new insights in the pathogenesis, and treatment of this severe disease. Funded: FAPESP and Federico Foundation.

Key words: *systemic sclerosis, collagen V, fibroblasts culture*

02.18- Estradiol Modulates the Synthesis and the Organization of ECM Components in Thyroid Cells

Michel Alexandre Villani Gantus, Leandro Miranda alves, Antonio Palumbo Júnior, Elaine Christina Lima de Souza, Tercia Rodriguez Alves, Denise Pires de Carvalho, Ana Maria Martinez Blanco, Luiz Eurico Nasciutti

1. UFRJ, UNIVERSIDADE FEDERAL DO RIO DE JANEIRO, Av. Pedro Calmon; Nº 550; Cidade Universitária ; Rio de Janeiro

The higher prevalence of thyroid disease in women suggests that estrogen (E2) might be involved in the pathophysiology of thyroid dysfunction. TS7 thyroid stromal cells, previously isolated and characterized in our laboratory, synthesize basement membrane components and express the cytokine transforming growth factor beta 1 (TGF- β 1). Under E2 stimulus, the secretion and activation of TGF- β 1 was increased. So, we hypothesized that the effects of E2 via TGF- β 1 synthesis and secretion on follicular thyroid cells could be mediated by extracellular matrix (ECM) secreted by stromal cells (paracrine action). Here, we investigated the effect of E2 on ECM secretion and assembly in co-culture assays of PCCL3 thyroid follicular cells and TS7 cells. We first demonstrated an increase of PCCL3 cell proliferation and a decrease

of iodide uptake, only when the cells were cultivated with TS7 cells, suggesting the involvement of the stroma in thyroid function. Furthermore, we observed that TGF- β 1 was able to decrease PCCL3 cell iodide uptake. On the other hand, when the cells were co-cultivated with TS7 cells, E2 and TGF- β 1 together stimulated the iodide uptake. Once is reported that TGF- β 1 enhance ECM remodeling, we analyzed the ECM components fibronectin (FN), laminin (LN), tenascin C (TNC), type IV collagen (COLIV), decorin (DEC) and chondroitin sulfate (CS) secreted by these cells stimulated with E2. Our results showed that E2 was able to modulate the synthesis and the organization of ECM components: CS and TNC in TS7 cells; FN, LN and CS in PCCL3 cells; DEC and CS in TS7/PCCL3 cell co-culture. These effects of E2 on thyroid cells could be related probably to TGF- β 1 pathway, suggesting a novel mechanism by which stromal-follicular interactions modulate thyroid function.

Keywords: *estrogen, extracellular matrix, stromal cells, TGF- β 1, thyroid*

02.19- Heparin Activates M3 Receptor to Promote Rat Aortic Ring Relaxation through Integrin Interaction

Edgar Julian Paredes Gamero¹, Fabio Augusto Moraes¹, Valquiria P. Medeiros¹, Giselle Zenker Justo¹, Eduardo HC Farias¹, Luiz Ivarne Dos Santos Tersariol¹, Edvaldo Da Silva Trindade², Guilherme I. Sassaki², Ana L. Lopes¹, Rosely O. Godinho¹, Antonio de Miranda¹, Alice Teixeira Ferreira¹, Helena Bonciane Nader¹

1. UNIFESP, Universidade Federal de São Paulo, R. 3 de maio, 100. 2. UFPR, Universidade Federal do Parana, 81531-990 Curitiba Paraná

Heparin is an established anticoagulant drug that has been widely used as antithrombotic agent since the 1940s. Furthermore, other pharmacological actions have been described for this glycosaminoglycan, including reduction of blood pressure in hypertensive humans, induction of nitric oxide production, and reduction vasoconstrictor endothelin-1 expression. In this study, the biochemical and pharmacological characteristics of heparins from different sources to induce vascular relaxation in rat aortic rings was investigated. The results indicated that these heparins are able to promote endothelium-dependent relaxation; however, with low affinity. Among the different heparins tested, unfractionated heparin extracted from bovine intestinal mucosa exhibited the maximal efficacy. Heparin extracted from bovine intestinal mucosa induces vascular relaxation and nitric oxide production by activation of the M3 receptor in rat aortic rings. Even though, no direct binding of heparin to endothelium was observed, fluorescent resonance energy transfer indicate an interaction between M3 receptor and integrin. In addition, pre-treatment of samples with the Src inhibitor (PP1) blocked heparin-dependent relaxation. Moreover, this effect is not related to either the molecular weight or the degree of sulfation of heparin molecules. On the other hand, the chemical reduction of carboxyl groups abolishes the relaxation effect of heparin. These results provide an insight into the molecular requirements and potential pharmacological actions of heparin in inducing vascular relaxation, opening a novel promising field of investigation on the potential use of heparin in medicine.

Keywords: *Heparin, Integrin, M3 receptor, Vascular smooth muscle*

02.20- Increased Cutaneous Collagen Gene Expression in Experimental Systemic Sclerosis

Soledad Elisabeth Bartolotti Matamoros¹, Ana Paula Pereira Velosa¹, Cristiane Carla de Oliveira^{1,1}, Maria Lúcia Hirata Katayama^{3,1}, Vera Lúcia Capelozzi¹, Natalino Hajime Yoshinari¹, Walcy Rosolia Teodoro¹

1. FMUSP, Rheumatology Division of the Faculdade de Medicina da USP, Av. Dr. Arnaldo 455, Cerqueira César. 2. FMUSP, Department of Pathology of the Faculdade de Medicina da USP, Av. Dr. Arnaldo 455, Cerqueira César. 3. FMUSP, Department of Radiology of the Faculdade de Medicina da USP, Av. Dr. Arnaldo 455, Cerqueira César

Background: Systemic sclerosis (SSc) is a rheumatic disease of unknown etiology characterized by vascular involvement, autoimmunity and skin and internal organs fibrosis. Skin thickening is the hallmark of the disease and the extent of cutaneous involvement has been shown to interfere in patient's abilities, activities, and outcome. **PURPOSE:** To evaluate collagen I, III and V distribution and gene expression in skin during the early and the late stage of scleroderma in the experimental model. **Methods:** To induce scleroderma, New Zealand females rabbits (n=12) were subcutaneously immunized with 1mg/ml of collagen V in complete Freund's adjuvant. At 30th day it was administrated an identical dose. Sequentially, at 45th and 60th day the animals were boosted intramuscularly with type V collagen in incomplete Freund's adjuvant. Skin biopsies were collected at 7 and 120 days after the first immunization and stained with hematoxylin-eosin, Masson's trichrome and Sirius red for morphological and morphometric analysis. The mRNA was isolated from fibroblasts culture and collagen V gene expression was evaluated through RT-PCR Real time. **Results:** An increase in collagen fibers was found in the dermis of animals corresponding to the immunized group at 7 days compared to controls ($9,72 \pm 3,41$ vs $6,54 \pm 0,94$; $p=0,05$) and 120 days ($65,47 \pm 0,94$ vs $20,69 \pm 5,60$ $p=0,01$). The analysis by RT-PCR Real time showed an increase in the mRNA expression of collagen V alpha-1 chain. The quantification was performed at 7($p=0,021$) and 120 days ($p=0,03$). **Conclusion:** The results obtained using this experimental model might be very important to a better understanding of the pathogenic mechanisms involved in the early stage of human SSc. Therapeutic protocols may lead in the future to promising treatments for SSc to avoid early remodeling of the skin.

Keywords: collagen V, sistemic sclerosis, collagen gene expression, skin

02.21- Real Time PCR: A New Excel-Based Data Sheet to Optimize Calculation of Reaction Preps and Automatically Analysis Efficiency Curves, Intra-Group Variation of Gene Expression for Selection of Endogenous Control and RQ Results by $\Delta\delta ct$ Method

Fabiana Kühne, Hernandes Faustino de Carvalho

1. UNICAMP, Universidade Estadual de Campinas, rua Charles Darwin, s/nº bloco N - sala 10 CEP 13083-863 - Campinas, SP

Real Time PCR (qRT-PCR) has been extremely useful as a tool for gene expression analysis, especially due to the sensitivity of the method. The necessity of using correct dilutions and preparation of the reaction components make difference in final results, and altering the preparation of the reaction among experiments or replicates may alter the final analysis. Aiming to minimize the time to calculate volumes and order of compounds to prepare reaction mixes, as well as to calculate the data, an Excel data sheet was developed. For planning the experiment, one informs the concentration of the sample in the reaction and in stock solution, the number of replicates, the number of genes tested and the number of variations in experimental model. The output is that the total number of reactions is informed, allowing the researcher to plan how many plates will be consumed in each experiment. Also, the volume of mixes and pre-mixes and the components needed, the volumes to dilute samples, primers and probes are calculated. After the run is completed, by copying and pasting the Cts from the csv file of run's exported results into the document, the intragroup variation of genes expression, the efficiency of the reaction between endogenous control and target gene, and gene expression by $\Delta\Delta C_t$ method using one internal control are calculated. The document is freely distributed by the authors.

Keywords: *real time PCR, endogenous control, gene expression, reaction calculation, data analysis*

02.22- Investigation of the Signaling Pathways Involved in the Cellular Response to Polymerized Laminin

Camila Hochman Mendez, Ana Carolina Giordani Duarte, Elisabete Freire da Cunha, Tatiana L. Coelho de Sampaio

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho

Laminin (LM), an extracellular matrix glycoprotein, plays a pivotal role in central nervous system (CNS) development. The laminin molecule, which is arranged in polymers *in vivo* and which can form artificial polymers *in vitro*, regulates several cellular functions in CNS, such as survival, proliferation, migration and differentiation. We have previously shown that neural cells can recognize and specifically respond to polymerized LM. The aim of this work was to investigate the intracellular pathways that mediate the effects induced by LM polymers. We produced artificial LM matrices polymerized at pH 4.0 (LM4) or at pH 7.0 (LM7), and analyzed retinal cells behavior *in vitro*. The rat retinal cell suspension was plated on the substrates LM4, LM7 or poly-L-ornithine (PLO). After 24 hours, the cells were fixed, immunolabeled for α -tubulin III and analyzed using phase-contrast or fluorescence microscopy. In order to investigate signal transduction, inhibitors of the kinases MAPK (PD98059), PKA (H89) and PKC (queleritrin chloride) were used, as well as Rolipram and Forskolin, substances that increase cAMP levels. These inhibitors were also used in cell proliferation assays. Our results show that on LM4 cells were arranged in monolayers where neurons emitted long processes evenly distributed over the substrate. On the other hand, cultures established on LM7 showed the formation of cell clusters, where cellular extensions were apparently confined to the clusters. Quantitative analysis revealed an increase of ~100% in the incorporation of thymidine when the cells were plated on LM4 when compared to LM7.

Treatment with H89, which blocks activation of PKA, decreased cell proliferation only on LM4. In contrast, inhibitors of PKC and MAPK did not affect proliferation on LM4. In addition, we showed that inhibition of PKA suppressed neurite outgrowth in LM4, while outgrowth was increased by Forskolin or Rolipram. Our data suggest that LM4 signaling in retinal cells involve activation of PKA. Supported by CNPq and FAPERJ.

Keywords: *differentiation, laminin, proliferation, signaling*

02.23- Folic Acid and Homocysteine Regulate the Microenvironment of the Murine Neural Crest Cells in Vitro.

Fernanda Rosene Melo, Andréa Gonçalves Trentin

1. UFSC, Universidade Federal de Santa Catarina, Campus Universitário - Trindade
Florianópolis SC

The neural crest NC is a transient structure of vertebrates embryos composed of highly pluripotent cells. The NC cells originate an extraordinary variety of cell types including neurons and glial cells of the peripheral nervous system, many endocrine cell types, and melanocytes. In addition, the NC of the cephalic region produces the greatest part of connective and skeleton tissues of the head and neck. The NC cells are highly responsive to the microenvironment. Folic acid (FA) deficiencies are associated with increased levels of homocysteine (HC) and implicated in many congenital abnormalities, such as neural tube closure defects and neurocristopathies which involves abnormal NC development. However, the effects of FA and HC on the morphogenetic processes of the NC are not well understood. Primary cell cultures were performed by mechanical dissection of 8.5 days mouse embryos neural tubes at the mesencephalic region. Explants were cultured on plastic dishes coated with fibronectin. After 48 hours the neural tubes were removed and the remaining emigrated NC cells were then trypsinized, and subcultured in the same culture condition for additional 10 days in the complex media containing HC (0, 75 to 300 μ M) or FA (22.5, to 90 μ M). Cell phenotypes were analyzed by immunofluorescence using the lineage-specific markers to glial cells (GFAP), neurons (β -III-Tubulin), smooth muscle cells (α SMA) and undifferentiated NC cells (p75). It was also analyzed the NC cell migration area, cell proliferation (by BrdU incorporation) and the proportion of apoptotic nuclei. The results demonstrated that HC significantly reduces the proportion of smooth muscle cells in a dose-dependent manner. This effect is prevented by the addition of AF. The proportion of neural and glial cells were not affected by the treatments. In addition, HC induced NC cell proliferation in a dose-dependent manner, effect prevented by the addition of FA. The proportion of apoptotic nuclei was insignificant in all tested condition. Taken together, our results demonstrate an important effect of FA and HC in NC differentiation and proliferation which may be involved in congenital anomalies, such as neurocristopathies.

Key words: *Neural crest, Homocysteine, Folic acid, Neurocristopathies*

03. ECM AND CELL GROWTH AND DIFFERENTIATION

03.1- Insulin-Like Growth Factor-I (Igf-I) Stimulate Migration of Murine Endothelial Cell Line in Vitro

*Maíra Estanislau Soares de Almeida², Iana Mayane Mendes Nicácio Viana¹,
Salette Smaniotto¹*

1. UFAL, Universidade Federal de Alagoas, Campus A. C. Simões - Av. Lourival Melo Mota, s/n -Tabuleiro dos Martins. 2. USP, Universidade de São Paulo, Av. Prof. Lineu Prestes-1524- Cid. Universitária

Insulin-like growth factors (IGFs) belong to a family of growth factors with structural homology to proinsulin. IGF-I is an important regulator of growth, survival, and differentiation in many tissues and can influence extracellular matrix (ECM) deposition. In addition, IGF-I stimulates repair of endothelial injury in cases such as atherosclerosis and may act as a stimulator of angiogenesis. The aim of this study was to investigate the effects of IGF-I on growth, production of ECM proteins and migration of endothelial thymic cells line (tEnd.1) in vitro. For the growth assay the endothelial cells were treated or not with IGF-I at different concentration (5, 10, 50 and 100 ng/mL) for 8 hours. A significant increase was observed in tEnd.1 growing depending on the concentration of IGF-I, when compared with control. Immunocytochemistry assay was carried up using anti-laminin, anti-fibronectin and anti-collagen IV antibodies. After washing cells were incubated with a second antibody. It was noticed an enhanced of fibronectin, laminin and collagen IV production by tEnd.1 cells treated with 100 ng/mL for 8 hours when compared to controls. Wound healing assay was performed using tEnd.1 confluent in six well plates. Wounds were generated with a sterile pipette tip. Cells were washed with PBS and treated or not with IGF-I (100 ng/mL). Photographs were taken at 0, 6, 12 and 24 hours. Analysis showed that tEnd.1 treated with IGF-I migrates faster during the time of the experiment, as compared to untreated controls. Cell migration was also evaluated using transwell culture inserts (8.0 mm pore size). Endothelial cells (1.0×10^6) were added to the upper chamber of the insert in 200 ml RPMI with or without IGF-I (100 ng/mL). Cells were allowed to migrate for 2, 4, 6 and 8 hours. When the endothelial cells were treated with IGF-I (100 ng/mL for 4, 6 and 8 hours), it was observed a significant increase in the number of migrating cells, compared to control. Our results show that IGF-I acts on endothelial thymic cell line (tEnd.1), improving growth, production of ECM and migration in vitro.

Keywords: *Cell Migration, Endothelial Cell, IGF-I, Angiogenesis*

03.2- WNT/B-Catenin Pathway Activation and Myogenic Differentiation are Induced by Cholesterol Depletion

*Debora Portilho, Mariana Senna, Fabio Mendes, Manoel Luis Costa, Jose Garcia,
Claudia Mermelstein*

1. UFRJ, Universidade Federal do Rio de Janeiro, CCS- Instituto de Ciências Biomédicas - Bloco F2 - sala 19

Skeletal muscle differentiation is a multi-step process that begins with the commitment of mononucleated precursors that withdraw from cell cycle. These myoblasts elongate while aligning to each other. This step is followed by cell fusion and the formation of long and striated multinucleated myotubes. Myogenic differentiation is influenced by a number of growth factors secreted for extracellular matrix, such Wnt proteins, which are required for the induction of embryonic myogenesis. Palmitoylation appears to be required for the secretion of Wnt. In the absence of the palmitoylation site, Wnt protein accumulates in the cells that synthesize it. Palmitoylated proteins are frequently targeted to cholesterol-enriched membrane domains. Recently, our group has shown that cholesterol depletion by methyl- β -cyclodextrin (M β CD) induces skeletal muscle differentiation and proliferation. The main interest of the present work was to study the effects of cholesterol depletion in the Wnt/ β -catenin signaling during muscle differentiation and whether this depletion could alter the Wnt availability. We used primary cultures prepared from breast muscles of 11-day-old chick embryos, and after 24 hours we treated it with M β CD. Cholesterol depletion increased the expression of β -catenin, its translocation to the nuclei, and activation of Wnt pathway. Frizz-1 enriched medium blocked the effects of M β CD. Moreover, myogenic cells grown with M β CD-conditioned medium and Wnt3a-enriched medium showed an enhancement in myogenic differentiation, as seen by the fusion index of myotubes. In addition, we analyzed the presence of Wnt3 in conditioned media. We found a more than 3-fold increase in the levels of Wnt3 in M β CD-conditioned medium when compared to control-conditioned medium. Our results show that Wnt/ β -catenin pathway is activated in the early steps of myogenic differentiation and can be regulated by membrane cholesterol. The addition of M β CD-conditioned medium led to an increase in fusion index of myogenic cultures in a similar way as M β CD by itself does, suggesting that the effects induced by M β CD could be caused by soluble factors present in the culture medium, such as Wnt molecules.

Keywords: *cholesterol, myogenesis, Wnt/ β -catenin*

03.3- Changes in the Expression of Adhesion Proteins after Membrane Cholesterol Depletion

Ana Claudia Batista Possidonio, Débora Morueco Portilho, Carolina Pontes Soares, Claudia dos Santos Mermelstein

1. UFRJ, Universidade Federal do Rio de Janeiro, ilha do fundão, Rio de Janeiro RJ

The formation of a skeletal muscle fiber begins with the withdrawal of committed mononucleated precursors from the cell cycle. These myoblasts elongate while aligning with each other, guided by recognition between their membranes. This step is followed by cell adhesion, fusion and the formation of long striated multinucleated myotubes. Interactions between myoblasts and the extracellular matrix (ECM) are very important since it can regulate both cell migration and adhesion of myoblasts. Actin, integrin, vinculin and paxillin are proteins involved in these interactions. Integrins are transmembrane glycoproteins that link the actin cytoskeleton to the ECM, and vinculin and paxillin are actin-associated proteins. Many plasma membrane molecules have been implicated in myoblast adhesion and fusion. One such molecule is cholesterol, a ubiquitous and abundant component of the

membrane that plays pleiotropic roles in plasma membrane function; it is involved in maintaining membrane fluidity and impermeability and the structure of lipid microdomains. Methyl-beta-cyclodextrin (MCD) can selectively removes cholesterol from cell membranes allowing the investigation of cellular and molecular mechanisms associated with cholesterol depletion. Recently, our group has shown that cholesterol depletion by MCD induces the proliferation and differentiation of chick-cultured myogenic cells (Mermelstein et al.,2005), and that Wnt/beta-catenin pathway is involved in these events (Mermelstein et al.,2006,Portilho et al.,2007). The main interest of the present work was to study the effects of cholesterol depletion in myoblast adhesion to ECM during myogenesis. We used myogenic primary cultures prepared from breast muscles of 11-day-old chick embryos, and after 24 hours we treated it with MCD at 4 mM for 30 minutes. Cell extracts with 28 and 48 hours were prepared for SDS-PAGE and immunoblotting. We also analyzed MCD treated-cells by indirect immunofluorescence to study the distribution of cell-ECM adhesion proteins. Our preliminary results show that MCD treatment induces a decrease in 30% in paxillin expression when compared to control cells. However, MCD treatment increases in approximately 20% the expression of vinculin. Our results shows that disorganization of microdomains induces changes in the expression of ECM adhesion proteins, suggesting that rafts could be involved in the adhesion, migration and fusion processes during the initial steps of skeletal muscle differentiation.

Keywords: *skeletal muscle, myogenesis, adhesion, Methyl-beta-cyclodextrin, membrane*

03.4- Bone Regeneration Using Biocomposite of Carbon Nanotube and Collagen Type I

Heloisa H M Della Colleta¹, Igor C F R Oliveira¹, Rodrigo Gribel Lacerda¹, Luiz Orlando Ladeira¹, Gregory Thomas Kitten¹

1. UFMG, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, ICB, Depto. Morfologia. 2. UFMG, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, ICEX, Depto. Física

The structural properties of carbon nanotubes provide them with characteristics that may be advantageous for numerous applications in biology and medicine. Biocompatible nanotubes have the potential to be used as molecular carriers capable of delivering drugs and other reagents to the inside of a cell, as physical support for artificial biological tissues, and possibly as stimulators of tissue regeneration. However, one major drawback which precludes the use of nanotubes is the difficulty in maintaining them in a desired, specific location. Therefore, we propose to include nanotubes in a gel consisting of native type I collagen. This biocomposite should provide a matrix which will restrict nanotubes in the desired location, plus also serve as a three-dimensional support for cells and tissues. The goal of the present project was to test the positive or negative effects of different concentrations of nanotubes associated with gels of type I collagen on the regeneration of alveolar bone. For these experiments, male Wistar rats were anesthetized and one upper molar on each side was extracted. The alveolar cavities were filled with a soluble mixture of collagen alone or collagen plus carbon nanotubes, both of which form a solidified gel *in situ* within minutes,

and then the rate of bone regeneration was compared to that seen in untreated control cavities. The animals were allowed to recover for 3, 7 and 14 days, after which the jaws were removed and fixed for histological analysis. The samples were examined both macroscopically and microscopically in order to determine the effects of nanotubes on bone regeneration. Macroscopic analysis evaluated the overall process of tissue regeneration, while microscopic analysis examined the morphology of the calcified area and the formation of new bone tissue. Immunohistochemical analysis showed that the nanotube-collagen biocomposites stimulate the expression of markers of osteogenesis, osteopontin and BMP-2, during the 3 and 7 day periods. However, after 14 days neither macroscopic nor morphological results showed any significant variations as compared to the controls. These results indicate that nanotube-collagen biocomposites do not interfere in the process of bone regeneration and provide a useful support for future studies.

Keywords: *Biocomposite, Carbon nanotube, Collagen type I, Extracellular matrix*

03.5- The Role Played by Collagen-I in the Compatibility of CP-Titanium Screws to Human Bone Cells

Lilian de Mello Gil, Fernando Costa e Silva Filho

1. UFRJ, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde - Instituto de Biofísica Bloco G sala G044

Due to their mechanical properties and corrosion resistance commercially pure (cp) Ti have been utilized for implants fabrication. In spite of the wide use of Ti for fabrication of dental implants, little is still known on the metal biocompatibility. Biocompatibility in turn, is a material property related to the acceptance of an artificial implant to the surrounding tissue and the body as a whole. It means, therefore, material biocompatibility is greatly dependent to what occurs at the cells-material interface and the mechanobehavior of this environment. Since the human bone extracellular matrix is a well known collagen rich one, we here explore the ability of human bone osteoblastic (HOB) cells to both associate and spread onto each one of uncoated and collagen I (COL)-coated dental mini-screws made of cp-Ti grade 4. Taken also in account that COL mechanics might influence the cytocompatibility of COL-coated Ti screws we used nested networks (3D) at 3mg/ml. HOB cells from confluent cultures were sequentially collected by centrifugation, rinsed with serum-depleted medium, counted (106cells/ ml), and then they were allowed to interact with the Ti screws. Such cell-Ti interaction process was followed by scanning electron microscopy and zymography. The morphology of cells in common contact with the screws (2D environment) was compared with cells soaked in collagen matrix (3D environment) both in contact with the screws using SEM. Images by SEM turns evident that cells have been involved by collagen matrix, and the cells morphology in contact with titanium do not differ from 2D and 3D environment. But comparing zymography results, a different band were found in the 3D condition. Tridimensional ambient seems to change the secretion of proteases by HOB cells, so, other studies should be designed with the perspective to elucidate cells behavior when in interaction with different biomaterials respecting the mechanical view. Mechanotransduction shows its importance in HOB cells behavior. As an in vitro biocompatibility assays are an important

way to found possible future damage to organisms tissues caused by biomaterials, the manner to work with cells should be as closer as possible than in nature. Supported by CNPq (a scholarship to LMG) and INPeTAm-Instituto Nacional de Pesquisa Translacional Integrando Saúde e Ambiente na Região Amazônica.

Keywords: *collagen, titanium, human osteoblastic cells, biocompatibility, 3D environment*

03.6- Extracellular Matrix Molecules Influence the Differentiation of Human Dental Pulp Putative Stem Cells

Viviane Aparecida Balvedi Polli, Beatriz Dalpont, Gustavo Coura, Marcio Silva Alvarez, Andrea Goncalves Trentin

1. UFSC, Universidade Federal de Santa Catarina, Campus Reitor João David Ferreira Lima – Bairro Trindade

In the human development, the origin of the dental pulp interactions between oral epithelium and ectomesenchymals tissues derivatives of cranial neural crest (NC). The NC consists of multipotent cells that differentiate into a wide variety of cells types including neurons, glial cells, melanocytes. In the head, the NC originates the mesectodermal derivatives including cartilage, bone and connective tissues and vascular smooth muscle cells (SMCs). The NC is influenced for different microenvironmental factors of migratory pathway, such as local growth factors and extracellular matrix (ECM). The ECM molecules, type I collagen (COL I) and fibronectin (FN), affect the migration of mesenchymal cells. These molecules have been identified in human dental development and in the NC cell differentiation. In this study, we established methods for cultivate human dental pup cells. Our results demonstrate that the cellular adhesion was significantly enhanced on COL I or FN than on plastic culture dishes. COL I and FN displayed similar values of cell adhesion although FN promoted increased cell dispersion. We identify in these cultures population of cells with NC characteristics. RT- PCR and immunohistochemistry revealed the presence of the NC stem cell markers p75 and nestin as well as markers of neurons (β -tubulin 3) and SMCs (α -smooth muscle actin, α SMA). This study provides evidence that human dental pulp cultures produce NC-like cells, suggesting a recapitulation of their embryonic state. These human dental structures revealed itself as a viable alternative source of primitive precursors to be used in stem-cell therapies. Supported by: PIBIC/UFSC, CNPq, CAPES, REUNI, MCT/INFRA, CNPq/PRONEX, FAPESC.

Keywords: *human dental pulp, Mesenchymal stem cell, extracellular matrix, putative stem cells, collagen I and fibronectina*

03.7- Extracellular Matrix Molecules Favours the Phenotypic Conversion of Neural Crest Derivatives

*Ezequiel Peyerl, Ricardo Castilho Garcez, Marcio Alvarez Silva,
Andrea Gonçalves Trentin*

1. UFSC, Universidade Federal de Santa Catarina, Campus Universitario - Trindade - Florianópolis

In the vertebrate embryo, the neural crest cells (NCCs) that migrate out from the neural primordium yield multiple phenotypes, including peripheral neurones and glia in the peripheral nervous system; cartilage, bone and connective cells in the head and vascular smooth muscle cells (SMCs) in cardiac outflow tract. Since NC cells are a migratory cell population, the differentiation of pluripotent NCCs is strongly directed by microenvironmental factors. A number of ECM molecules with permissive influences on NC cell migration, such as type I (CLI), type IV collagen (CLIV) and fibronectin (FN) have been identified in NC cell migratory pathways. In addition, ECM proteins have also been suggested to influence NC cell differentiation. It was previously demonstrated in the quail NC, some examples of phenotypic conversion. Pigment cells and glial Schwann cells can convert into each other in vitro when exposed to the mitogenic signal of endotelin-3 and, Schwann cells can be converted into smooth muscle cells both in vitro and in vivo. In the present work, we provide an in vitro analysis of the influence of ECM molecules on the developmental potential of avian SMCs. We have focused our attention on α -smooth muscle actin (α SMA)-positive cells of NC origin. Thoracic aortic-smooth muscle cells expressing α SMA, isolated from quail embryos, were plated in plastic culture dishes coated with FN (50 μ g/ml) or CLI (50 μ g/ml). The 24 hours-primary-culture was composed essentially of α SMA+ cells. These cells progressively lose their α SMA marker and progressively express β -III-Tubulina (β -III-Tub) and Schwann myelin protein (SMP) markers. On cells were cultured on CLI, significantly higher proportions of neurons (β -III-Tub+ cells) as well as glia (SMP+ cells) were observed. Taken together our results show evidences for the acquisition of a neuronal and glial phenotypes by NC-derived-smooth muscle cells and suggest that the differentiation state of NC-derived cells is unstable and capable of reprogramming. Supported by: MCT/CNPq, CAPES, FAPESC.

Keywords: *differentiation, glia, Neural crest, Neuron, smooth muscle cells*

03.8- Quail Neural Crest Cell Differentiation: The Effects of Fgf2 and Microenvironment

*Denise Avani Bittencourt^{1,3}, Meline Coelho Costa^{6,8}, Giordano Wosgrau Calloni^{2,1,4},
Marcio Alvarez Silva^{1,2,8}, Andréa Gonçalves Trentin^{1,2,3}*

1. UFSC, Universidade Federal de Santa Catarina, Campus Universitário Trindade - Florianópolis, SC. 2. CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Setor Bancário Norte - Brasília, DF. 3. CNPQ, Conselho Nacional de desenvolvimento Científico e Tecnológico, Brasília, DF. 4. FAPESC, Fundação de Apoio a

Pesquisa Científica e Tecnológica de SC, Florianópolis, SC. 5. MCT/INFRA, MCT/INFRA, Brasília, DF. 6. PIBIC/UFSC, Programa Institucional de Bolsa de Iniciação Científica, Campus Universitário Trindade - Florianópolis, SC. 7. REUNI/UFSC, Reestruturação e Expansão das Universidade Federais, Campus Universitário Trindade - Florianópolis, SC. 8. CNPQ/PRONEX, CNPQ/PRONEX, Brasília, DF

The neural crest (NC) is an embryonic structure originated at origin from the edges between the neural plate and the epidermis during the neurulation process. The cells of the NC (CNC) are composed of populations of precursors already determinate and pluripotent cells capable to originate a variety of derivatives, including neurons and glial cells of the peripheral nervous system, melanocytes, endocrine and glandular cells, skeletal and connective tissues of the head and neck. The development of the CNC involves the segregation of some multipotent progenitors of an initial population of migratory cells. The microenvironment in which NC cells migrate and differentiate have a greatly influence in the phenotype that they express. In this work, we investigate the effects of the FGF2 in the progenitors differentiation/potentialities of the CNT in cultures coated with collagen type I (COL I) or on a monolayer of 3T3-NIH mouse fibroblasts. The neural tubes of quail embryos were dissected at the trunk level (15-16 HH stage) and cultivated in a medium containing α -mem medium enriched with, bovine fetal serum and chicken embryonic extract. After 24 h, the explants were removed and the remaining cells were subcultured with FGF2 on COL I-coated dishes or on 3T3NIH. Cells were treated with FGF2 during 6 days and additional 4 days in a complex medium of the differentiation medium. Cultures were analyzed in the presence of cell phenotypic markers: myelin anti-protein (Schwann cells); anti- β III-Tubulin and tyrosine hydroxylase (neurons); anti- α -smooth muscle actin (smooth muscle cells); melanocyte/melanoblast early marker (melanocytes). Our results demonstrate that FGF2 stimulates the proliferation of the NC cells of the CNT in cultures cultured on COL I and increases the expression of the phenotypic markers, except for the melanocytes. NC cells cultures on 3T3-NIH fibroblasts displayed increased proportions of neurons and glial cells. Taken together, our results demonstrate that the removal of FGF2 treatment followed by NC cell culture in conditions of cellular differentiation on 3T3-NIH fibroblasts in differentiation medium increases the NC differentiation to glial and neuronal phenotypes at the expenses of the melanocytes and smooth muscle phenotypes. Therefore, FGF2 has a central role in NC differentiation programs that is dependent of the microenvironment.

Key words: *Neural Crest, Differentiation, FGF2, Microenvironment, Quail*

03.9- Morphological Behavior of IEC-6 Epithelial Cells Grown in 2D and 3D Matrices Made of Type I Collagen

Thelma Simões de França Maciel, Lilian de Melo Gil, Gustavo Conde Menezes, Débora Barreiros Petrópolis, Fernando Costa e Silva Filho

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, CCS-Bloco G sala G-044, Cidade Universitária

The extracellular matrix (ECM) is a proteinaceous meshwork which in turn, may serve as a scaffold for cell growth, proliferation, survival and differentiation. Collagen (COL) is the most abundant component of the animal ECM. It is formed by an association of COL fibrils, which results from the polymerization of monomeric COL units. Depending on the organization and the resulting supramolecular structure of such fibers, the ECM can create specific microenvironments within animal tissues. The two-dimensional (2D) arrangement of the fibrils formed by COL I (monomeric COL I) on plastic surfaces provides a more rigid substrate for cells, while the three-dimensional (3D) one (fibrillar COL I) provides a more flexible, porous and irregular substrate. Much of mammalian cells respond differently to each one of 2D and 3D COL I, being that such cell responsiveness may be followed by structural and biochemical tools. Here, we did try to characterize some properties concerning IEC-6 (rat small intestinal epithelial) cells grown in a twenty-four multiwell plate which have been coated or not with COL I: biofilms (2D) and fibers (3D). 10^5 , $5 \cdot 10^5$ or 10^6 IEC cells/ml were allowed to interact for 24, 48, and 72h at 37°C with uncoated and COL I-coated surfaces which in turn, have been constituted at 2D or 3D. Cell morphology was followed by both light and scanning electron microscopies. As expected, cells exhibited different morphologies in 2D or 3D environments. At 2D cultures, cells appeared smaller than at 3D. After 24h of incubation in 2D (in absence or presence of COL biofilm) we did not observed any remarkable difference among cells grown onto uncoated and COL I-coated surfaces. By contrast, cells cultured on 3D appeared stretched and most of them appeared to be aligned with the COL fibers. The cells exhibited large cytoplasmic expansions after 48h of incubation, and they also tended to associate themselves, not forming monolayers. The molecular mechanisms that underlie such differential cell behavior are currently under investigation. Supported by CNPq-PIBIC, CNPq-UNIVERSAL, and INPeTAM.

Keywords: *IEC-6, morphology, three-dimensional matrix, type I collagen*

03.10- Characterization of Hair Follicle Stem Cells Cultured on Type I Collagen Matrix

Ricardo Mueller Cesario Pereira, Bruna Hilzendeger Marcon, Juliana Klein Zucco, Andrea Golçalves Trentin, Márcio Alvarez

1. UFSC, Universidade Federal de Santa Catarina, Campus universitário Trindade - Florianópolis, SC, Brasil

The adult mammalian hair follicle (HF) contains a population of epidermal stem cells in a specific niche, the bulge area. The HF stem cells are multipotent and act in the support, grown and development of HF, epidermis and sebaceous gland. However, it has been recently identified in the bulge of HF, the presence of cell with neural crest (NC) characteristics. The NC is a transient embryonic structure that originates in the neural folds. NC cells invade the embryo, and differentiate into a wide array of cell types and tissues. NC derivatives include neurons and glial cells of peripheral nervous system, endocrine cells, myofibroblasts, pigment cells, as well as bone and cartilage. In the present study, we established a protocol to cultivate HF cells from 60 day-mice whiskers. HFs were dissected and cultured directly in the plastic dishes or on type I collagen (Col-I) coated dishes during 21 days in the presence of Alfa-

MEM supplemented with chicken embryo extract, fetal calf serum and growth factors and hormones (T3, insulin, glucagon, hidro cortison, transferrin, EGF and FGF 2). In order to perform a molecular characterization of these cell cultures, we analyzed by RT-PCR the mRNA expression of the following phenotypic markers: NC stem cells or their derivatives (PAX-3, FoxD3, Sox10, P0, SMA), epidermal phenotypes markers (cytokeratins 5, 14, 15, 19) embryonic stem cell (OCT-4) and HF cells (CD 34, CD 200 and). We used HF dissected of adult mice (6-8 weeks old) from vibrissa region and put in 35mm wells culture dishes. With we observed that Col-I matrix were more efficient in promoting HF attachment, cell adhesion, migration and proliferation than the plastic. Furthermore, the addition of growth factors and hormones induced the expression of the NC markers. In conclusion, we established a cell culture protocol that was effective to isolate NC-like cells from the adult mouse whisker follicle. The addition of growth factors and hormones was effective in stimulate the expression of NC-derived cell-markers, and was also favorable to the maintenance of NC-like stem cells. HF stem cells are promising candidates for cell therapy due to their high plasticity and accessibility in the adult mammalian skin.

Keywords: *Hair follicle, Cell culture, Collagen type I*

03.11- Experimental Study of the Fracture Components in Bone under Dynamics Loads

Moyses Messias Souza de Sant'Anna, Thaís Cristina Mendes da Silva, Luiz Alberto Batista, Liszt de Oliveira Palmeira, Jorge Jorsé de Carvalho

1. UERJ, Universidade do Estado do Rio de Janeiro, Boulevard 28 de Setembro, 87, fundos, Vila Isabel

Basic scientific and clinical investigations show bone tissue responds to certain patterns of loading by increasing matrix synthesis and, in many instances, by changing the composition, organization, and mechanical properties of their matrix. This study is a proportional application of dynamic loads in callus mending the structure components of the extracellular matrix, the cell proliferation index and the differentiation process accelerating the regeneration of bone tissue, considering the direction, the magnitude and frequency of the load. Eighty male Wistar rats, age 3 months and average weight of 270 Kg were used. Tritiated thymidine was applied one hour before the fracture. The animals were divided for each time of fracture, respectively, 12hs, 1, 2, 3, 4, 6, 8 and 10 days after the fracture. The fracture in the right tibia were held with a standardized instrument for closed fracture and controlled by X-ray. the protocol was approved by the Ethics Committee in accordance with the principles adopted by the COBEA. The sample were prepared and processed by histological, immunohistochemical and autoradiography technique. The results of the radiography confirmed the effectiveness of standardized fracture equipment developed to maintain the fracture alignment. the hematoxylin-eosin stained images, show large areas of cartilaginous cells migrating to form the callus two days after the fracture. Also, the Weigert resorcin-fuchsin histochemical technique with prior oxidation, revealed the presence of a vast network of oxytalan fiber in the pre-cartilage in the areas of cell proliferation two days after the fracture. there was no immune-staining for PCNA in animals with 12 hs after

fracture. However, there was reasonable immune-staining from one day after the fracture and a strong immune-staining starting 2 days until the 10 days after the fracture, suggesting intense cell proliferation. The results of the sections stained with Picro Sirius red technique and observed through a polarization microscope did not show collagen fibers after 12 hs of fracture. However, 2 days after the fracture there was moderate and strong staining of collagen fibers with picro Sirius Red technique. The results of the immune-staining technique for TGF- α demonstrated strong distribution in the hematoma area 12 hours after the fracture until 10 days after fracture in the callus.

Keywords: *Bone Fracture, Dynamics Loads, Extracellular Matrix, Cell Proliferation*

04. ECM AND INFLAMMATION AND IMMUNOLOGY

04.1- Cardiac Inflammatory Response in Experimental Duchenne Dystrophy

Cynthia Machado Cascabulho, Vinicius Cotta de Almeida, Andrea Henriques Pons

1. FIOCRUZ, Fundacao Oswaldo Cruz, Av Brasil, 4365 Manguinhos - RJ

Duchenne muscular dystrophy (DMD), a severe X-linked inflammatory myopathy caused by mutations in the dystrophin gene, is characterized by progressive degeneration of skeletal and cardiac muscles. The mdx mouse, a strain presenting spontaneously mutant dystrophin gene, has become widely used as a model for DMD and presents distinct and sequential phases of the disease, namely, myonecrosis, regeneration and fibrosis. This work aimed to evaluate, in the cardiac muscle, the migration of cellular subpopulations with possible effector/regulatory roles that might correlate to the progression of the disease and reveal possible important regulatory pathways. Tissues were collected from mdx mice at stages of myonecrosis (6 weeks after birth) and regeneration (12 weeks after birth). Histopathological analysis showed mostly macrophages and fibroblasts composing the inflammatory cardiac foci, and mast cells, which are mostly immature and present in pericardium by the age of 2 weeks. However, these cells apparently migrate to endocardium while acquiring staining-based mature characteristics, peaking at 6 weeks after birth. FACS analysis of collagenase-dissociated tissue, from mice at the myonecrosis stage showed a lymphocyte population comprised mostly of CD3+CD4+ cells, but also with CD3+CD8+ cells and a progenitor population of Sca1+Mac1-CD4-CD8- cells. Further phenotyping of this population revealed them as Sca+B220+CD117- cells. However, at the regeneration phase they were depicted as Sca+B220+CD117+ cells. We also found about 12% of $\gamma\sigma$ + T cells within the lymphocyte population, although in the blood they were less than 1%. Interestingly, in 12-week old mice, lymphocytes were barely found in cardiac tissue, and this coincides with strong down-regulation of CD62L on blood lymphocytes. In conclusion, we observed the infiltration of different inflammatory cells into the heart at different stages of the disease, and ongoing functional in vitro assays aim to analyze migratory capability of these cells.

Key words: *Duchenne muscular dystrophy, inflammation, CD62L*

04.2- The Role of Galectin-3 In Homeostasis of B Lymphocytes and in the Structure of Lymphoid Organs

Camila Brand, Felipe Leite de Oliveira, Radovan Borojevic, Christina Maeda Takiya, Márcia Cury El-Cheikh

1. UFRJ, Universidade Federal do Rio de Janeiro, AV. Carlos Chagas Filho, 373 - Ilha do Fundão

Introduction: Galectin-3 is a beta-galactoside binding protein involved with several physiological and pathological processes, such as cell activation, proliferation, differentiation, migration, apoptosis and tumoral progression. The role of this lectin is unclear in the lymphohematopoietic cell biology, although it was demonstrated that its absence interferes with B lymphocyte compartment, accelerating the plasma cell generation during *Schistosoma mansoni* infection (Oliveira *et al.*, 2007). **Objectives:** In this study, we evaluated B lymphocytes in their different compartments, as well as analyzed the lymphoid organs involved in homeostasis of these cells, like spleen and bone marrow (BM), in galectin-3 deficient mice (Gal-3 ^{-/-}). **Methodology:** Male C57/bl6 and Gal-3 ^{-/-} mice were sacrificed 60-90 days after birth. For histological analysis, formalin-fixed tissue sections were deparaffinized and stained with hematoxylin and eosin. For immunohistochemistry, sections were processed with standard reagents, followed by incubation of anti-B220 or anti-CD138 antibodies, detected with biotin/avidin-peroxidase reaction. By flow cytometry, B cells were defined by anti-B220 FITC and anti-IgM Biotinylated antibodies. **Results:** The histological analysis revealed an alteration of spleen structure in gal-3^{-/-} mice, presenting larger disorganized lymphoid follicles and heterogeneous sizes. B220 staining was increased in gal-3^{-/-} mice, mainly in red pulp. The same profile was observed for CD138 staining, showing higher numbers of plasma cells in these animals. In the BM, we observed significant differences such as reduced number of granulocytes and mononuclear cells and atypical distribution of megacariocytes compared with wild type mice. In addition, we observed an increase in B220⁺ IgM⁺ cells (immature B cells) in peripheral blood of gal-3^{-/-} mice. **Conclusions:** Gal-3 interferes with histological aspects of lymphoid tissues, modulating their hematopoietic functions. The increase of B220⁺ cells in the spleen could indicate an accelerated efflux of these cells from the BM to complete the terminal differentiation into plasma cells (CD138⁺). The large number of B220⁺ IgM⁺ cells in the peripheral blood, as well as the decreased number of total BM cells suggested that gal-3^{-/-} regulates the organization of microenvironments related with B cell development. **Support:** CNPq.

Keywords: *Spleen, B Lymphocyte, Plasma Cell, Galectin-3*

04.3- Effect Of Exogenous Galectin-1 on Leukocyte Migration: Modulation of Cytokine Levels and Adhesion Molecules in Experimental Inflammation

Cristiane Damas Gil¹, Caio Eduardo Gullo¹, Sonia Maria Oliani²

1. FAMERP, São José do Rio Preto School of Medicine, Av. Brigadeiro Faria Lima 5416; 15090-000, São José do Rio Preto-SP, Brazil. 2. IBILCE-UNESP, Instituto de

Biociências, Letras e Ciências Exatas, Rua Cristóvão Colombo 2265, 15054-000, São José do Rio Preto-SP, Brazil

Galectins are a conserved family of glycan-binding proteins, usually found in the extracellular matrix, that have emerged as pleiotropic regulators of innate and adaptive immune responses. Some members of the galectin family, including galectin-1 (Gal-1), can regulate immune cell trafficking, activation, cytokine secretion, and apoptosis by triggering multivalent interactions with cell surface glycoconjugates. In this study we focus the effect of galectin-1 (Gal-1) on the expression of integrin (CD11b) and L-selectin (CD62L) during leukocyte migration. C57BL/6 mice received i.p. injection of zymosan (1.0 mg) in 0.5 ml of sterile saline, whereas control animals were injected with an equal volume of saline. Another experimental group was treated with human recombinant Gal-1 (hrGal-1; 0,3 µg per animal) before zymosan administration. At 4 and 24h later, animals were sacrificed and peritoneal wash, blood and mesentery were processed for light and electron microscopy analysis. Cytokine levels of peritoneal fluid were analyzed by ELISA and expressions of CD62L and CD11b were monitored in blood neutrophils (PMN) by flow cytometry and mesenteric PMN by immunocytochemical analysis. Zymosan peritonitis provoked expected signs of inflammation at 4h, including significant increase in extravasated PMN in mesentery and peritoneal wash, mirrored by blood neutrophilia. These changes subsided after 24h. Pharmacological treatment with hrGal-1 produced a significant reduction of PMN recruitment at 4h in all tissues analyzed compared to non-treated animals. In this early phase, the anti-inflammatory effects of hrGal-1 were associated with inhibition of the TNF- α and IL-1 β levels in the peritoneal fluid. Immunocytochemical analysis showed a co-localization with Gal-1 and adhesion molecules CD11b and CD62L in plasma membrane of mesenteric PMN. The addition of hrGal-1 resulted in an increased expression of CD62L in blood and mesenteric PMN, but diminished the expression of CD11b in PMN during the transmigration process. Our results indicate an important mechanism of anti-inflammatory action of Gal-1 through modulation of pro-inflammatory cytokines and firm adhesion induced by CD11b on PMN recruitment, and might have an impact on the development of new strategies for inflammatory diseases.

Keywords: *galectin, integrin, neutrophil, selectin, zymosan peritonitis*

04.4- Induced Asthma by Oxidative Stress in Balb/C Mice

Renata Tiscoski Nesi¹, Manuella Lanzetti Daher de Deus¹, Frank Silva Bezerra², Tatiana Almeida Pádua¹, Mariana Barcellos de Ávila², Clarissa Bichara Magalhães², Walter Araújo Zinn², Débora Souza Faffé², Samuel dos Santos Valença¹, Luis Cristóvão de Moraes Sobrinho Porto¹

1. UERJ, Universidade do Estado do Rio de Janeiro, Avenida Professor Manuel de Abreu, nº444, 3º andar, Rio de Janeiro. CEP 20550-17. 2. UFRJ, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Av. Carlos Chagas Filho, 373, Rio de Janeiro, RJ

Most of the factors that result in oxidative stress are known. However, the correlation between these mechanisms and the pathophysiology of asthma is still unclear. The present

study investigated the involvement of oxidative stress in asthma. BALB/c male mice were used and divided into two groups (n = 40 each) that received either 10% of ovalbumin by subcutaneous injection (OVA group) or phosphate buffer (Control group) for 4 intercalated days. After seven days, the groups were challenged with 1% of nebulized ovalbumin for 20 minutes during 5 consecutive days. On the 21st day, animals were euthanatized. Homogenized lungs were prepared for biochemical dosages for protein, enzymatic assays for Catalase (CAT), superoxide dismutase (SOD) and Glutathione peroxidase (GPx), neutrophil elastase, metalloproteinase (MPO) and malondialdehyde forms (MDA) dosages were done. Also, TIMP-2, MMP-9 and 12 expressions were measured (n=20 each). The content of protein increased 55% in the OVA group (P<0.01). The CAT activity 26% was decreased in the OVA group (P<0.05). SOD activity was also decreased in the OVA group (36% P<0.05). However, GPx activity was increased 142% in the OVA group (P<0.001). The OVA group showed a 21% reduction in the activity of MPO (P<0.05). The level of MDA increased 47% in the OVA group (P<0.001). The levels of nitrite were 21% reduced in OVA group (P<0.05). Although there was an increase of HMGB-1 expression in the OVA group, the expression of neutrophil elastase was lower. The expression of TIMP-2 was similar in both groups, however, the gelatinolytic activity of MMP-2 and -9 increased in the OVA. These data indicated that oxidative stress is an initial factor of the inflammatory process of asthma, promoting the growth of proteolytic activation. This study helps understanding the potential involvement of redox unbalance is the precursor of changes in asthma.

Keywords: *asthma, allergy, Balb/c mice, inflammation, oxidative stress*

04.5- Collagen V-Induced Nasal Tolerance in Experimental Systemic Sclerosis Downregulates Cutaneous Remodelling

Natália Borsonello, Ana Paula Velosa, Antonio Santos Filho, Angela Batista Gomes dos Santos, Maria Roseli Callado, Margarete Vendramini, Cleonice Bueno, Edwin Roger Parra, Vera Lúcia Capelozzi, Natalino Hajime Yoshinari, Walcy Rosolia Teodoro

1. FMUSP, Faculdade de Medicina da Universidade de São Paulo, Avenida Doutor Arnaldo 455 - Cerqueira César

Introduction: Systemic sclerosis (SSc) is an autoimmune disease of the conjunctive tissue characterized by vascular damage, autoantibodies presence, collagen deposit and fibrosis in the skin and internal organs. Collagen V (Col V) has been described as potential allo /auto-antigen in animal models. Recently, our group described an SSc experimental model in health rabbit induced by Col V immunization. **Objective:** Our aim was to study skin remodeling and autoantibody production in the experimental model of Scleroderma (SSc), following nasal tolerance with human Col V. **Methods:** Female New Zealand rabbits (N=12) were immunized with two doses of 1mg/ml of Col V in complete Freund's adjuvant and additional two boosters in incomplete Freund's adjuvant to induce SSc. After 150 days, half of from these immunized rabbits were submitted to type V collagen-induced tolerance receiving a daily nasal administration of 25mg of Col V. Control animals (N=6) were only submitted to type V collagen-induced tolerance. Serial skin biopsies were performed at 0, 150 and 210 days, and stained with HandE, Masson's trichrome and Picrosirius for morphological and morphometric

analysis. Types I, III and V collagen, TGF β and PDGF were identified by immunofluorescence. The animal's sera samples were collected in the same times to determine anti types I, III, IV and V collagen, anti-Scl-70 and antinuclear antibodies (ANA). **Results:** Skin biopsies from immunized animals confirmed SSc morphology as previously described, such as progressive decrease of papillary dermis, appendages atrophy, increased type I, III and V collagen deposition and augmented expression of TGF β and PDGF. Rabbits with Col V-induced nasal tolerance showed reduction of skin involvement, with significant decrease of total collagen amount ($p=0.002$), and types I ($p<0.01$), III ($p<0.01$) and V ($p<0.01$) and lower TGF β and PDGF expressions. Humoral immune response did not change with nasal tolerance. **Conclusions:** Col V nasal tolerance promotes regression of skin remodeling process in the experimental model of SSc. We suggest that nasal tolerance with type V collagen can be a promising therapeutic option to treatment scleroderma patients. *Funded by FAPESP, CNPq, Frederico Foundation, LIMs 17 and 05*

Keywords: *Systemic Sclerosis, Nasal Tolerance, Collagen V, cutaneous remodelling, autoantibodies*

04.6- April Modulates in Vitro Thymocyte Migration and Cell Adhesion by Interacting with Hspg

Cecilia Rocha¹, Désio Aurélio Farias de Oliveira¹, Leticia Fernandez², Salete Smaniotto³, Déa Maria Serra Villa-Verde¹, Michael Hahne², Wilson Savino¹, Carla Eponina de Carvalho Pinto⁴

1. FIOCRUZ, Fundação Oswaldo Cruz, Av. Brasil 4365, Mangueiras - CEP 21045-900.
2. IGMM, Institut de Génétique Moléculaire de Montpellier, 1919 route de Mende 34293 Montpellier cedex 5.
3. UFAL, Universidade Federal de Alagoas, Praça Afranio Jorge s/n, Prado - CEP 57010-020.
4. UFF, Universidade Federal Fluminense, Outeiro de São João Batista s/nº, Valonguinho - CEP 24210-15

Proteins of the tumor necrosis factor (TNF) family play an important role in many biological processes like cell proliferation, differentiation, survival and death. APRIL (A Proliferation-Inducing Ligand) is a member of this cytokine family, promotes tumor proliferation and survival, and modulates B cell activities, enhancing cell survival and antibody production. However, whether APRIL exerts any effect on T cells remains unclear. APRIL acts by interacting with two receptors, BCMA and TACI, both expressed on lymphocytes. Additionally, APRIL binds to cell surface heparan sulphate proteoglycans (HSPG). Transgenic mice overexpressing APRIL develop a B-1 cell neoplasia. In preliminary analysis, we observed by immunohistochemistry, an increase in B220+ cells in the thymus from APRIL transgenic mice compared to the controls. The thymus is a central lymphoid organ in which T cell differentiation occurs. This process is deeply dependent on thymocyte migration throughout the organ, and for that the thymocytes interact with the thymic epithelial cells (TEC) and extracellular matrix molecules such as fibronectin and laminin. APRIL effects in the thymus remain unknown. This work aimed to study the effect of APRIL on thymocyte migration of normal mice. Firstly, we investigated whether APRIL could modulate thymocyte survival and proliferation. We did not find any alterations in response to in vitro

treatment with APRIL recombinant protein. Even though, the *ex vivo* migration assays revealed a chemorepulsive effect probably owing to a decrease on thymocyte adhesion as showed by co-culture with TEC. Interestingly, these results did not correlate with any difference in the membrane expression of fibronectin or laminin receptors (respectively VLA-4 / VLA-5, and VLA-6) on thymocyte subpopulations. APRIL chemorepulsive effect was enhanced by CXCL12 and laminin (but not by fibronectin). Besides, differing from TEC, thymocytes did not express typical APRIL receptors. Nevertheless, pre-treatment with heparin partially blocks migration, suggesting that the effect of APRIL on thymocyte migration is mediated by HSPG interaction. In conclusion, APRIL seems to stimulate thymocyte migration, and downregulates cell adhesion, by interacting with HSPG. Financial support: CNPq, FIOCRUZ/INSERM.

Keywords: APRIL, HSPG, MIGRATION, THYMOCYTE

04.7- TGF Beta and Collagen I and V Alfa-1 Chains Mrna Decrease by Collagen V-Induced Nasal Tolerance in Lung of Systemic Sclerosis Model

ELLEN GAMES JACOB PEREIRA¹, Ana Paula Pereira Velosa², Edwin Roger Parra², Maria Lucia H. Katayama³, Vera Luíza Capelozzi², Natalino Hajime Yoshinari¹, Walcy Rosolia Teodoro¹

1. FMUSP, Rheumatology Divison of the Faculdade de Medicina da USP, Rua Dr. Arnaldo, 455 Cerqueira Cesar. 2. FMUSP, Pathology Department of the Faculdade de Medicina USP, Rua Dr. Arnaldo, 455 Cerqueira Cesar. 3. FMUSP, Labortory of Oncology of the Faculdade de Medicina USP, Rua Dr. Arnaldo, 455 Cerqueira Cesar

Purpose: Systemic sclerosis (SSc), or scleroderma, is a rheumatic disease characterized by autoimmunity, vasculopathy and interstitial fibrosis in skin and several internal organs. Pulmonary manifestations of this disease normally are causes of disease-related morbidity and mortality. To evaluate the inflammatory process, collagen deposition, mRNA collagen synthesis, TGF- β expression in the pulmonary tissue in model of SSc after COLV-induced nasal tolerance. **Methods:** Female New Zealand rabbits (N=12) were immunized with 1mg/ml of Col V emulsified in Freund's complete adjuvant, followed by two extra immunizations with Freund's incomplete adjuvant (IM). Nasal tolerance was induced in a group of six IM animals, 150 days after the beginning of immunization and was treated for 60 days, receiving a daily nasal administration of 25mg of Col V (IM-TOL). Control animals (N=6) were only submitted to type Col V-induced tolerance (CT). The inflammatory cells and TGF- β expression in vessel and septal interstitium were evaluated by point counting method. For evaluation of collagen amount was used software Image Pro-plus 6.0 in immunofluorescence microscopy and types I, III and V collagen alfa-1 mRNA expression by Real-time PCR. **Results:** IM-TOL, when compared to IM, presented decrease lymphocytes (4.33 ± 1.71 vs. 11.45 ± 2.52 , $p < 0.05$), macrophages (5.74 ± 2.27 vs. 7.66 ± 1.57 , $p < 0.05$) and monocytes (1.92 ± 0.73 vs. 27.67 ± 3.72 , $p < 0.05$) as well as significant reduction in collagen content around the small vessels (0.37 ± 0.12 vs. 0.87 ± 0.28 , $p < 0.001$) and bronchioles (0.29 ± 0.14 vs. 0.65 ± 0.17 , $p < 0.001$). The lung tissue of IM-TOL, when compared to IM, showed decrease immunostaining of types I, III and V collagen, reduced alfa-1 mRNA expression to types I

(0.10 ± 0.07 vs. 1.0 ± 0.53 , $p=0,002$) and V (1.12 ± 0.42 vs. 4.74 ± 2.25 , $p=0,009$) collagen. The TGF- β expression in lung vessel endothelium cells ($10.77 \pm 4,3$ vs. $43.5 \pm 5,7$, $p<0,0001$; IM vs. IM-TOL) and pulmonary fibroblasts (9.93 ± 3.77 vs. 53.68 ± 3.77 , $p<0,0001$; IM vs. IM-TOL) decreased in IM-TOL. Conclusions: COL V-induced nasal tolerance in the experimental model of SSc decreased the inflammatory process and regulated the pulmonary remodeling process, inhibiting TGF- β expression, collagen deposition and collagen I and V gene synthesis. Additionally, the tolerance with COL V can be a promising therapeutic option for human scleroderma treatment. Funded by FAPESP, CNPq, Federico Foundation, LIMs 17 and 05

Keywords: *COLLAGEN V, NASAL TOLERANCE, SYSTEMIC SCLEROSIS MODEL, PULMONARY, TGF BETA*

04.8- The Role of Laminin and CXCL12 in T-Cell Migration During Cardiac Allogeneic Graft Rejection

Ingo Riederer, Ariany Oliveira Santos, Daniella Areas Mendes-da-Cruz, Suse Dayse Silva-Barbosa, Wilson Savino

1. FIOCRUZ, Fundação Oswaldo Cruz, Av Brasil nº4365- Manguinhos.Caixa-Postal: 906

Rejection is an immune process caused mainly by Major Histocompatibility Complex (MHC) differences between donor and recipient. During rejection, recipient T cells are activated in secondary lymphoid organs and migrate to the graft in order to destroy it. Cell and extracellular matrix (ECM) contact causes many important biological events, and ECM proteins are involved in the rejection process. Using a non-vascularized model of allogeneic heart transplantation, where a heart from a neonatal is transplanted subcutaneously in the ear of an adult mouse, we have previously demonstrated a correlation between the cellular infiltrate, and the ECM. Moreover, the treatment of the heart graft with an anti-laminin (LN) antibody (a major ECM protein), reduced the cellular infiltration to the graft. It's also known the importance of chemokines driving T cell migration during immune response, specifically the chemokine CXCL12 influencing naive T cell migration. In the same model, we decided to study the relationship of the T cell population with LN and CXCL12 in the graft draining lymph nodes (cervical draining lymph nodes - CDLN) and the graft. We observed an enhancement of cell numbers, mainly CD8+, expressing VLA-6 (LN receptor) in high densities, in CDLN from transplanted animals. We also observed the presence of CXCL12 in CDLN, both in transplanted and control. However, an increased deposition of LN was detected in transplanted CDLN. Moreover, we verified the migratory ability of T cells obtained from CDLN from transplanted and control animals through functional analyzes, based on a transmigration assay using LN as a substrate. This experiment revealed that T cells of transplanted animals had an increased migratory ability towards LN when compared with controls. However, a higher migration was observed when we placed cells to migrate towards LN combined to CXCL12. Our study unravels the importance of LN and CXCL12 in the circuit graft/draining lymph nodes, and reinforce the importance of T cell migration in the rejection process.

Keywords: *Laminin, CXCL12, Migration, Graft rejection, Lymph nodes*

04.9- In Vivo Mast Cells Regulation by Stem Cell Factor and Death Receptors after *Trypanosoma Cruzi* Infection

Marcelo Meuser-Batista¹, José Raimundo Correa¹, Marcos Meuser¹, Maurilio Soares³, Francisco Farias-Filho¹, Patricia Machado e Silva¹, Robson Coutinho Silva², Andrea Henriques-Pons¹

1. FIOCRUZ/IOC, Instituto Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro (RJ). 2. IBCCF, Instituto de Biofísica Carlos Chagas Filho, Rio de Janeiro, Rio de Janeiro (RJ). 3. IBMP, Instituto de Biologia Molecular do Paraná, Curitiba (PR)

Although mast cells (MC) are pivotal in inflammatory and fibrotic etiologies, their role in *Trypanosoma cruzi*-induced cardiomyopathy is unexplored. Most studies are restricted to MC quantification in the heart and association with fibrosis. In this work, we studied cardiac and peritoneal MC in *T. cruzi* infected mice and observed a reduction of 60% in the number of MC in both compartments after infection. The reduction of cardiac MC numbers was mainly due to apoptosis of pericardial mucosal MC (MMC) and this death is possibly associated with a primary decreased in the local stem cell factor (SCF) production. Through RT-PCR we observed that the infection induced the expression of P2X7 and Fas in cardiac MC, two molecules reported to be involved in cell death and inflammatory regulation. Using *gld/gld* mice we observed that Fas/Fas-L interaction can be responsible for MC apoptosis induced by the infection in the heart, but not in peritoneal cavity. Conversely, infected P2X7^{-/-} mice indicated that this purinergic receptor induces MC death in peritoneum. These data illustrate the complexity of molecular interactions controlling inflammatory cell function and indicates that MC not only influence the inflammatory response, but are also under the constraint of the inflammatory milieu in different compartments. *Financial support:* CNPq, Fundação Oswaldo Cruz, PRONEX FAPERJ.

Keywords: *Mast cells, P2X7, Fas, Chagas Disease, Inflammation*

04.10- Collagen Type V Distribution in Rheumatoid Arthritis (RA) Induced by Methylated Bovine Serum Albumin (Mbsa)

Silvana Atayde, Antônio Santos Filho, Natalino Yoshinari, Suzana Mello, Walcy Teodoro
1. FMUSP, Faculdade de Medicina Universidade São Paulo, Av Dr. Arnaldo, 455

Collagen type V Distribution in Rheumatoid Arthritis (RA) induced by methylated Bovine Serum Albumin (mBSA) Background: RA is an autoimmune disease, characterized by chronic sinusitis in peripheric articulations. Inflammatory infiltrate and pro-fibrotic cytokines induce the synthesis of extracellular matrix components, especially collagen. We developement the autoimmune disease model that present over expression and remodeling of type I, III and V collagen (COL V) in synovial tissue. Purpose: To examine the (COL V) distribution in the synovial tissue in experimental RA after 45 days after (RA) induced by methylated bovine serum albumin (mBSA) Methods: Arthritis was elicited in males Lewis rats (n=6), weighting 250g, by subcutaneous injection of methylated bovine serum albumin

(mBSA) emulsified on Freund complete adjuvant into the right knee. After 7 days it was administrated an identical doses and at 14th day the animals were boosted with 500 μ g of mBSA diluted in saline solution (RA). As control, NaCl 0,9% was injected in the left knee of the animals (CT). After 45 days the synovial tissue were collected, maintained in 10% formaldehyde and used HandE, Masson's trichrome and Picrosirius staining to morphological analysis, and COL V expression by immunofluorescence. Results: After 45 days of RA, evaluation the rat's right knee articulation was characterized by pronounced inflammatory cells in the synovial tissue, pannus formation and destruction of cartilage and bone in relation to rat's control articulation. In RA it was observed a synovial membrane and vessel wall thickness, besides substitution of subsynovial adipose tissue by collagen fibers with a fine and heterogenic pattern, characterizing a chronic disease phase. Immunostaining demonstrated COL V higher expression around the vessels as well in the subsynovial reparation tissue. Conclusions: The increase of subsynovial tissue COL V expression in arthrogenic rats suggests that this protein can be present during the inflammatory process evolution in human disease.

Keywords: *Collagen type V, Rheumatoid Arthritis, autoimmune disease model, synovial tissue, inflammatory process*

04.11- Characterization of the Novel Systemic Sclerosis Model Induced by Rabbits Type V Collagen Immunization

Ana Paula Pereira Velosa¹, Solange Carrasco¹, Antonio Santos Filho¹, Maria Roseli Callado¹, Maria Lúcia Hirata Katayama², Edwin Roger Parra³, Vera Luíza Capelozzi³, Natalino Hajime Yoshinari³, Walcy Rosolia Teodoro¹

1. FMUSP, Rheumatology Division of the Faculdade de Medicina da USP, Av.Dr. Arnaldo, 455. 2. FMUSP, Department of Radiology of the Faculdade de Medicina da USP, Av.Dr. Arnaldo, 455. 3. FMUSP, Department of Pathology of the Faculdade de Medicina da USP, Av. Dr. Arnaldo, 455

BACKGROUND: Systemic sclerosis (SSc) is a rheumatic disease of unknown pathogenesis, characterized by endothelium lesion, autoantibodies production, collagen deposition in skin and internal organs. Experimental models have been used to understand SSc pathogenesis, but none reproduce all manifestation found in this disease. Recently, it was developed a rabbit autoimmunity model by immunization with type V collagen (COL V), a peculiar fibril with high antigenicity. **PURPOSE:** To characterize the morphological and immunological changes and organs involvement in rabbits immunized with COL V and to compare with the features found in human SSc. **METHODS:** New Zealand female rabbits (n=20) were subcutaneously immunized with 1mg/ml of collagen V in complete Freund's adjuvant. At 30th day it was administrated an identical dose. Sequentially, at 45th and 60th day the animals were boosted intramuscularly with type V collagen in incomplete Freund's adjuvant. Control groups (n=20) were immunized with Freund's adjuvant in the same way. To morphologic study, the serial skin biopsies were collected at 0, 7th, 15th, 30th, 75th and 120th day, and samples of the internal organs at 75th (n=10) and 120th (n=10) day. Collagen mRNA expression was evaluated by immunostaining and RT-PCR-RealTime. Sera were

analyzed by ELISA, immunoblotting and immunofluorescence. RESULTS: Morphologic analysis demonstrated progressive skin fibrosis with increased collagen expression in cutaneous fibroblasts; pulmonary vasculitis with cellular infiltration, increased collagen deposition and vessels thickness leading to vessel obliteration; and systemic involvement with collagen deposit in esophagus, heart, kidney and synovia. It was found endothelial damage and vascular lesion in skin and internal organs. Immunologic analysis showed presence of autoantibodies, such as anti-Scl-70, anti-endothelin, antinuclear antibodies (ANA) and anti-collagen I, III and IV antibodies. A peculiar finding was the expression of unusual COL V fibrils pattern in the analyzed tissues. CONCLUSION: The same organs normally affected in patients with SSc are compromised in COL V immunized rabbits. The development of progressive scleroderma-like lesions, such as vascular compromising, fibrosis and presence of autoantibodies, like Scl-70, a specific antibody, demonstrated that it is a multifactorial SSc model, necessary to understand the pathology of the disease.

Keywords: *Collagen V, Systemic sclerosis, autoimmunity, experimental model*

04.12- Endothelial Dysfunction in Experimental Model of Systemic Sclerosis

Roberta Gonçalves Marangoni¹, Ellen Games Jacob Pereira¹, Edwin Roger Parra², Ana Paula Pereira Velosa¹, Vera Luiza Capelozzi², Natalino Hajime Yoshinari¹, Walcy Rosolia Teodoro¹

1. FMUSP, Rheumatology Division of the Faculdade de Medicina da USP, Av. Dr. Arnaldo, 455. 2. FMUSP, Department of Pathology of the Faculdade de Medicina da USP, Av. Dr. Arnaldo, 455

Purpose: The aim of the present study was to investigate endothelial dysfunction in an experimental model of SSc induced by collagen V (COLV). **Methods:** Female rabbits from New Zealand lineage were immunized with COLV plus Freund's adjuvant as previously described (Pathol Res Pract 200;681, 2004). Animals immunized only with Freund's adjuvant were used as controls. The animals were sacrificed two hundred and 10 days after the first immunization. Endothelial dysfunction was evaluated by endothelin-1 (ET-1) immunoexpression in lung. **Results:** Endothelial dysfunction evaluated by ET-1+ endothelial cells was strongly increased in COLV ($87.66 \pm 2.11\%$; $p=0.001$) compared to controls ($11.20 \pm 2.39\%$; $p=0.001$) in lung sections, respectively. **Conclusion:** Endothelial dysfunction was found in a rabbit model of SSc induced by collagen V, emerging as a promissory model for future investigations of vascular remodeling. **Support:** Federico Foundation, FAPESP 07/59791-6, FAPESP 07/59792-2

Keywords: *Systemic sclerosis, Collagen V, Experimental model, endothelium*

04.13- Acidic-Polymerized Laminin Modulates Acute Inflammation in Spinal Cord Injuries.

Marcos Assis Nascimento, Karla Menezes, Gabriel Gomes Maia, Claudia Farias Benjamim, Aurélio Vicente Graça de Souza, Tatiana Lobo Coelho de Sampaio

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Brigadeiro Trompowsky, s/n.
Centro de Ciências da Saúde, Ilha do Fundão

In spinal cord injury the primary damage is followed by events that expand the initial injury. The exacerbated inflammatory response is responsible for demyelination and neuronal apoptosis, which contributes to the formation of a microenvironment non-permissive to axonal regeneration. In previous studies, we verified that laminin polymerized in acidic buffer was capable of increasing neuritogenesis *in vitro*. In addition, the application of these polymers to the injured cord led to a significant functional recovery as early as 24 hours post-lesion, which suggests containment of the inflammatory damage. In order to confirm such anti-inflammatory effect of the polymers Wistar rats were subjected to partial transection and received 10µl injections of acidic buffer or acidic laminin immediately after the lesion. The animals that received acidic laminin showed macrophage infiltrates only at the lesion epicenter, while the control group showed macrophage positive staining all over the spinal cord extension. Furthermore, the serum levels of C reactive protein, an acute inflammatory process marker, was reduced in 50% in the first 24 hours. In the same period, the number of neutrophils in the lesion epicenter was 61% smaller in the treated group (27,3+/-4,5 vs. 10,6+/-1,7). Our data suggest that laminin polymerized in acidic buffer showed an immunomodulatory effect, helping to preserve the nervous parenchyma and facilitating the regenerative process.

Keywords: *Inflammation, Laminin, Spinal Cord Injury*

04.14- Loxosceles Intermedia Sphingomyelinase D Induces the Activation of Proteases on Human Erythrocyte Membrane, Able to Hidrolise Fluorescence Substrates

Alessandra Veloso de Melo, Fernanda Calheta Vieira Portaro,
Denise Vilarinho Tambourgi

1. IBu, INSTITUTO BUTANTAN, AV Vital Brazil, 1500, Butanta - Sao Paulo

Introduction: Spiders of the *Loxosceles* genus, also known as “brown spiders”, are distributed worldwide. Accidents caused by these spiders are associated with severe clinical symptoms, including dermonecrotic lesions, thrombosis, vascular leakage and, eventually, intravascular hemolysis and renal failure, that can be lethal. In Brazil, three major species, implicated with human envenoming, are responsible for more than 6.000 cases of envenomation each year. The main toxic component present in the venom is a sphingomyelinase D (SMase D). *Loxosceles* SMase D promotes the activation of membrane bound proteinases on erythrocytes and nucleated cells (Tambourgi et al, 2000; van den Berg

et al, 2002). In the case of erythrocytes, this led to an increased susceptibility to activation of complement (C), via the alternative pathway, because of proteinase-induced cleavage of glycophorins, which are important regulators of C-activation (Tambourgi et al, 2000). Objectives: The aim of the present study was to further investigate the activation mechanism of the membrane-bound proteinase(s), responsible for the cleavage of the erythrocyte glycophorins, induced by SMases D. Methods: We have used the fluorescence resonance energy transfer (FRET) substrate Abz-FRSSRQ- EDDnp as a tool to explore the proteolytic activity of human erythrocyte membranes. Membranes were incubated with 5 mM of the substrate, in the presence or absence of a recombinant SMase D from *L. intermedia* (named as P2 - 5 µg) at 37°C, in a 96 well plate. The hydrolysis reaction, of the fluorogenic peptide, was monitored during 40 minutes in a spectrofluorimeter. In addition, inhibition assays were performed in order to determine the class of the activated proteinase(s). Results: Data obtained show that the erythrocyte membrane preparation have a basal proteolytic activity upon the fluorogenic peptide. However, treatment with SMase P2 induces the increase of this enzymatic activity in approximately 20%. Moreover, the use of elastase inhibitor was able to reduce the membrane proteolytic activity, induced by treatment with P2, in about 40%. Conclusion: Our results suggest that SMase P2 is able to activate proteinase(s) on erythrocytes membrane preparations and that elastase maybe involved in the process.

Keywords: *enzymatic activity, erythrocyte membrane, fluorogenic substrate, proteases, sphingomyelinase D*

04.15- Galectin-3 Regulates Thymocyte Homeostasis

Ednéa Oliveira de Abreu, Tiago Dutra Pereira Ramos, Danielle Silva dos Santos, Wilson Savino, Déa Maria Serra Villa Verde

1. FIOCRUZ, Fundação Oswaldo Cruz, Av. Brasil, 4365, Manguinhos, CEP: 21045-900, Rio de Janeiro, RJ, Brasil

Galectin-3 belongs to a family of beta-galactoside-binding proteins highly conserved throughout animal evolution. It is found at different subcellular compartments, modulating biological processes such as cell adhesion, migration, proliferation and apoptosis. In the thymus, this endogenous lectin is found in the cortex and the medulla, being produced by thymic epithelial cells, phagocytes and thymocytes. Galectin-3 interacts with glycoproteins on the cell surface and in extracellular matrix proteins (ECM), such as fibronectin and laminin. We showed that galectin-3 plays a de-adhesive role in the thymus, modulating interactions between thymocytes and the thymic microenvironment. Galectin-3 null mice represent a useful tool for the study of the role of this lectin in thymus physiology and pathology. Accordingly, we used these animals to further approach the influence of galectin-3 in thymocyte homeostasis. We initially observed that thymus weight and cellularity were significantly decreased in galectin-3 null (gal-3^{-/-}) mice when compared to strain/age/sex-matched wild type animals. This led us to investigate spontaneous thymocyte proliferation, evaluated by 3H-thymidine incorporation. Gal-3^{-/-} thymocytes showed a decreased proliferative ratio when compared to wild type thymocytes. Moreover, cytofluorometric analysis of thymocyte subpopulations in gal-3^{-/-} mice showed a decrease in the percentage

and absolute numbers of CD4+CD8+ cells. We also observed a decrease in the expression of extracellular matrix receptors (VLA-4, VLA-5 and VLA-6), as well as the CXCR4 chemokine receptor in gal-3^{-/-} thymocytes, which are important molecules for thymocyte migration during the process of intrathymic differentiation. Our data suggest that galectin-3 is a relevant regulator of thymocyte homeostasis, with likely implications in thymopoiesis.

Keywords: *extracellular matrix, galectin-3, thymus*

04.16- Galectin-3 is Probably Related to the Thymic Alterations Observed in Nod Mice

Tiago Dutra Pereira Ramos¹, Carla Eponina Carvalho Pinto²,
Déa Maria Serra Villa Verde¹

1. FIOCRUZ, Fundação Oswaldo Cruz, Av. Brasil, 4365, Manguinhos, CEP: 21045-900, Rio de Janeiro, RJ, Brasil. 2. UFF, Universidade Federal Fluminense, Outeiro de São João Batista, s/n, Campus do Valonguinho, Centro, Niterói, RJ

Galectin-3 belongs to a family of endogenous lectins which bind to beta-galactosides presented on the cell surface and extracellular matrix glycoproteins. It is involved in biological functions such as cell growth, adhesion, proliferation and apoptosis. Moreover, galectin-3 is found in several tissues and organs, being highly conserved among animal species. Recent papers related galectin-3 to autoimmune diseases such as insulin-dependent diabetes mellitus. This disease is characterized by pancreatic beta-cell destruction by T cells and auto-antibodies. NOD (non-obese diabetic) mice represent a suitable model to study diabetes, as they develop diabetes in a similar way as humans. Previous data from our group showed important thymic alterations in these mice with the development of giant perivascular spaces, intermingled with extracellular matrix network, suggesting a deficiency in the exit of mature thymocytes from the organ. This defect is associated with low expression of the fibronectin receptor VLA-5. Considering that galectin-3 is a de-adhesive protein recently related to the pathogenesis of type I diabetes, the aim of this study is to evaluate the participation of this lectin in the alterations observed in NOD thymus during the development of diabetes. In order to attend this objective, we evaluated galectin-3 expression in the thymus by immunohistochemistry; transwell thymocyte migration in the presence of galectin-3 towards fibronectin, laminin and CXCL12; and adhesion of galectin-3-treated thymocytes onto fibronectin and laminin. We showed an apparent increase in galectin-3 expression in the thymic medulla of NOD mice. In addition, preliminary data on NOD thymocytes treated with galectin-3 showed a decreased migratory and adhesive capacity towards fibronectin when compared to control thymocytes. However, under laminin and CXCL12 stimuli they presented an increased migratory response. Our data suggest that galectin-3 is able to modulate thymocyte interactions with the thymic microenvironment, probably contributing to the arrest of thymocytes in giant perivascular spaces as found in the thymus of NOD mice.

Keywords: *extracellular matrix, galectin-3, insulin-dependent diabetes mellitus, thymus*

05. ECM AND HOST-PARASITE INTERACTIONS

05.1- Effect of a Fucosylated Chondroitin Sulfate in Experimental Cerebral Malaria and in Parasite Cytoadherence

Marcele Fontenelle¹, Yara Carollo Blanco¹, Cristina Pontes Vicente², Stefanie Costa Pinto Lopes¹, Mauro Sérgio Gonçalves Pavão³, Paulo Antonio de Souza Mourão³, Cláudio Chrysostomo Werneck², Fabio Trindade Maranhão Costa¹

1. UNICAMP, Universidade Estadual de Campinas, Deptº de Genética, Evolução e Bioagentes, IB, C.P.6109, Campinas, SP. 2. UNICAMP, Universidade Estadual de Campinas, Deptº de Bioquímica, IB, Campinas, SP. 3. UFRJ, Universidade Federal do Rio de Janeiro, Deptº de Bioquímica, Instituto de Ciências Biomédicas, Rio de Janeiro, RJ

Severe malaria is characterized by the sequestration of *Plasmodium falciparum*-infected erythrocytes (IEs) in vital organs. Adhesion of IEs to endothelial cells has a key role in the pathogenesis of life-threatening malaria and could be targeted by antiadhesion therapy. Sulfated polysaccharides, like heparin, have been shown to inhibit cytoadherence of parasitized erythrocytes to host receptors to different extents. Heparin was previously used in the treatment of severe malaria, but it was discontinued due to the occurrence of serious side effects such as intracranial bleedings. We showed that fucosylated chondroitin sulfate (FucCS), a polysaccharide isolated from sea cucumber, *Ludwigothurea grisea*, which is similar to heparin, composed of a chondroitin sulfate backbone substituted at the 3-position of the β -D-glucuronic acid residues with 2,4-disulfated α -L-fucopyranosyl branches, is a potent inhibitor of the IEs cytoadhesion to human lung endothelial cells (HLEC). Inhibition seems to be nonspecific and occurs in a concentration-dependent-manner. Furthermore, treatment with FucCS at 1 mg/Kg/animal/day showed to improve survival of C57BL/6 mice infected with *Plasmodium berghei* ANKA, an experimental model for cerebral malaria and characterized by a potent inflammatory process. Of note, treated mice did not exhibit visible side effects during therapy. Thus, we suggest FucCS as a promising candidate for adjunct therapy to prevent severe malaria outcomes. This project is supported by CNPq.

Keywords: *Fucosylated Chondroitin Sulfate, Cerebral malaria, Severe malaria, parasite cytoadherence*

05.2- Regulation of Extracellular Matrix Expression and Distribution in Trypanosoma Cruzi -Infected Cardiomyocytes

Cláudia Magalhães Calvet, Francisco O. R. de Oliveira Jr., Tânia C. de Araújo-Jorge, Mirian Cláudia de Souza Pereira

1. Fiocruz, Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ

Extracellular matrix (ECM) alterations have been demonstrated in Chagasic cardiomyopathy. ECM enhancement in the heart is detected together with inflammatory infiltrates and elevated TGF- β and TNF- α levels in plasma, which may indicate that

inflammation process participates actively in this event. However, the mechanisms underlying ECM alterations still need to be investigated, and poor attention has been given to the specific response of infected cardiomyocytes concerning ECM expression. Cardiomyocyte primary cultures obtained from mouse embryos were infected with *T. cruzi*, Y strain. After 24h of infection, the cultures were treated with recombinant TGF- β (1, 2, 5, 10 and 15 ng/ml) and TNF- α (50 and 100 ng/ml) for 48h. The localization of fibronectin (FN) and laminin (LM) was visualized after 72h of infection by indirect immunofluorescence. For quantitative analysis of ECM expression, total protein extract from cytokine treated cultures was separated by electrophoresis, transferred to nitrocellulose membrane, and ECM expression was detected through western blot procedure. Confocal laser microscopy showed that treatment of cardiomyocytes cultures with TGF- β and TNF- α leads to an enhancement of FN matrix only in uninfected cardiomyocytes, while infected myocytes displayed low FN expression. Digital image analysis also revealed low superposition of FN signal with parasite nests in cytokine treated and untreated cultures. Cytochalasin D treatment demonstrated that microfilaments disarray induced a disturbance in the FN network of cardiomyocytes, suggesting that cytoskeleton disruption caused by *T. cruzi* infection disorganizes FN matrix. Additionally, western blotting analysis revealed a 2-fold increase in the FN expression in cardiomyocyte cultures after cytokines treatment, whereas *T. cruzi* infection significantly reduced FN levels in all conditions. In contrast, no change in the LM expression was detected after cytokines treatment by western blotting. LM distribution was altered in *T. cruzi*-infected cardiomyocytes, showing intense LM labeling only at the cells periphery even after cytokine treatment. Therefore, our data demonstrate that TGF- β and TNF- α stimulates FN expression only in uninfected cells of the *T. cruzi*-infected cultures, whereas the cells harboring the parasites presents low or no FN fibrils. Supported by FIOCRUZ, FAPERJ, CNPq, PAPES

Keywords: *Cardiomyocyte, Fibronectin, TGF-beta, TNF-alpha, Trypanosoma cruzi*

05.3- Differential Effect of TGF-B on Extracellular Matrix Remodeling of Trypanosoma Cruzi -Infected Cultures

Tatiana Araújo Silva, Cláudia Magalhães Calvet, Mirian Cláudia de Souza Pereira

1. LUC/IOC/FIOCRUZ, Lab. Ultra-Estrutura Celular/IOC/ Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ

Extracellular matrix (ECM) components are involved in the pathogenesis of the chronic phase of Chagas' disease, TGF- β , a cytokine related to ECM stimulation, is implicated with chagasic fibrosis. Based on the fact that high levels of TGF- β are detectable in the plasma of chronic chagasic individuals [1] and the low responsiveness of cardiac cells to TGF- β stimulus in vitro [2], we evaluated the effect of TGF- β treatment on the ECM remodeling after infection of different cell types with *T. cruzi*. Mouse embryo cardiomyocyte primary cultures (CM), fibroblasts from cardiac muscle (FMC) and a skeletal myoblast lineage (L6E9) were infected with *T. cruzi* (Y strain) and treated for 48h with recombinant TGF- β (1, 5, 10 and 15 ng/ml). The spatial distribution and expression of fibronectin (FN) was analyzed by confocal laser scanning microscopy and western blot assay after 72h of infection, respectively. Confocal microscopy analysis revealed enhancement of FN signal in CMs

stimulated with doses higher than 10ng/ml of TGF- β , as previously demonstrated [2]. Curiously, a dose 10 folds lower of TGF- β (1ng/ml) mediated augment of FN fibril formation in L6E9 cells and FMC. In contrast, *T. cruzi* infection induced the lack of fibrillar FN matrix in CM and L6E9, while uninfected cells in the *T. cruzi*-infected culture presented staining profile of FN similar to control. Remarkably, FN disorganization in highly infected cells was visualized even after stimulation of the cultures with high doses of TGF- β (10-15 ng/ml). Preliminary data from protein expression revealed a raise of 2.39 and 3.35 folds in FN levels in L6E9 stimulated with 1 ng/ml and 10 ng/ml of TGF- β , respectively. In CMs, only an elevated dose of TGF- β (15ng/ml) induced a maximum raise of 2 fold in FN expression, suggesting that CM are less responsive to TGF- β treatment than skeletal mioblasts and cardiac fibroblasts concerning FN expression. This way, our data open new perspectives to evaluate alterations in TGF- β signaling and the mechanisms that result in ECM reduction in *T. cruzi* infected cells, including cytoskeleton alterations and receptors modulation. [1] Araújo-Jorge et al, 2002, J Infect Dis, 186(12):1823 [2] Calvet et al, 2009, Int J Med Microbiol, 299(4):301. Supported by CNPq, FAPERJ, FIOCRUZ, PAPES V.

Keywords: *Fibronectin, TGF- β , Trypanosoma cruzi*

05.4- Analysis of Ecm Elements in Experimental Paracoccidioidomycosis Wound Healing Induced by Hene Laser

Rosaria Di Gangi¹, Jacy Gameiro², Patricia Resende Alo Nagib², Liana Maria Verinaud²

1. PUC-Campinas, Pontificia Universidade Católica de Campinas, Av. John Boyd Dunlop, s/n°. 2. UNICAMP, Universidade Estadual de Campinas, Cidade Universitária "Zeferino Vaz"

Introduction: Paracoccidioidomycosis is a systemic mycoses highly prevalent in Brazil caused by a thermally dimorphic fungus, *Paracoccidioides brasiliensis*. Its clinical forms vary from cutaneous to systemic forms. Conventional methods of treatment are uncomfortable and last long time. Previous studies in our group have demonstrated the positive capacity of the HeNe laser to accelerate the wound healing process and to slow down the fungi dissemination. Here we have demonstrated the alterations in laminin and fibronectin induced by treatment with HeNe laser. **Material and methods:** 8 week-old male mice foot pad were inoculated with *Paracoccidioides brasiliensis*. After one week, the foot pad were treated with 3J/cm² HeNe laser and then collected and submitted for immunofluorescences staining with specific antibodies to laminin and fibronectin. **Results:** HeNe Laser treatment has an influence on the density of ECM elements analyzed with quantitative and qualitative differences between treated and non-treated lesions. Statistics analysis show the following data to laminin non-treated 71.93 ($\pm$ 16.08) and treated 60.26 ($\pm$ 16.26) and, to fibronectin non-treated 54.72 ($\pm$ 7.80) and treated 35.75 ($\pm$ 10.00). When compared to treated lesions, non-treated lesions have a major density of both laminin and fibronectin ECM elements. **Conclusions:** The slower density of ECM elements in the treated lesions shows that the wound healing process has already been finished when comparing to non-treated lesions which enhances the capacity of HeNe laser to accelerate the wound healing process.

Keywords: *Paracoccidioidomycosis, Laser HeNe, ECM, Wound Healing*

05.5- Role of P38 Mapk and Mlck in the Transendothelial Migration of the Pathogenic Fungus *Sporothrix Schenckii*

Paula Magalhães Souza Deccache¹, Camila Castro Figueiredo¹, Maria Augusta Arruda²,
Leila Maria Lopes-Bezerra¹, Verônica Morandi¹

1. UERJ, Universidade do Estado do Rio de Janeiro, Departamento de Biologia Celular, LabAngio, PHLC, sala 203. 2. UERJ, Universidade do Estado do Rio de Janeiro, Departamento de Farmacologia

Sporotrichosis is a subcutaneous mycosis caused by *Sporothrix schenckii*. In immunocompromised hosts, this fungus may invade the bloodstream and disseminate to tissues such as lungs and bones. We demonstrated that TGF-beta 1 induces transendothelial migration of *Sporothrix schenckii* by a paracellular route. Here, we investigated the role of a few signaling pathways related to cell contraction in *S. schenckii* transendothelial migration. We analyzed the effect of genistein (tyrosine kinase inhibitor), SB 203580 (a p38 MAPK inhibitor) and W7 (MLCK inhibitor) in this process. Endothelial cells (EC) grown on 3 µm-porous inserts were pretreated with the inhibitors, before their 6-hour interaction with yeast cells. Genistein significantly inhibited (63%) transendothelial migration of yeast cells. We further verified that the activation of p38 MAPK and MLCK is required for fungus transmigration across EC since this event was inhibited (71% and 60% respectively) by SB 203580 and W7. Since in previous works we observed that *S. schenckii* leads to an increase in tyrosine phosphorylation of a 125 kDa EC protein, we investigated the role of integrin-dependent FAK (focal adhesion kinase) phosphorylation in *S. schenckii* transendothelial migration, by immunoprecipitation and western blotting analysis. We verified that FAK phosphorylation is not altered by the interaction yeasts with EC. We concluded that *S. schenckii* transendothelial migration seems to be modulated by tyrosine and serine/threonine-phosphorylation events. The passage of the fungus across EC triggers a signaling transduction cascade that involves p38 MAPK and MLCK activation. We also showed that FAK activation is not associated with this process. Supported by CNPq, CAPES and FAPERJ.

Keywords: *Adhesion, Cell Signaling, Endothelial Cells, Migration, Sporothrix schenckii*

05.6- The Mechanochemical Interaction between the Parasitic Protozoan *Entamoeba Histolytica* and 3D Collagen I Matrices

Débora Petrópolis¹, Maria Aparecida Gomes², João Viana², Marcia Attias¹, Nathan Viana¹, Bruno Pontes¹, Fernando Costa Silva-Filho¹

1. UFRJ, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil. 2. UFMG, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Trophozoites of the human parasite *Entamoeba histolytica* recognize and bind to extracellular matrix (ECM) components including collagens, fibronectin and laminin-1. Data underlie the occurrence of a bidirectional signal of mechanochemical nature exchanging from ECM to parasites and from parasites to ECM. Both chemistry and mechanics concerning the meshwork formed by ECM triggers indeed the activation of many cell functions, including invasiveness. We explore herein the mechanochemical interaction between trophozoites of *E. histolytica* and tridimensional (3D) matrices made of collagen I (COL). *E. histolytica* trophozoites (strain HM1:IMSS) were cultured in each one of 2D (immobilized at 50 and $\mu\text{g ml}^{-1}$ COL) and 3D collagen. Cultivation of the protozoa at 3D was performed at the following COL densities (mg/ml): 1,5 (low), 3,0 (medium), and 5,0 (high) mg.ml^{-1} , being that COL was polymerized for 1 h at 37°C . Just after, the resulting COL matrices were rinsed, and allowed to interact with parasites for 24h and 48h (invasion assay). Alternatively, parasites were associated to COL during its polymerization (interaction assay). Followed by scanning electron microscopy (SEM) we observed that parasites induced a profound architectural rearrangement in the networks formed by COL. Also at SEM (field emission mode) we observed thin cytoplasmic projections emerging from trophozoites to COL surface at 2D. Such cytoplasmic expansions were not observed among parasites during interaction with COL fiber at 3D. Zymograms were carried out in order to investigate proteases releasing by parasites during their cultivation at 3D. The resulted zymograms clearly showed an indirect correlation between COL density and amount of proteases release by the parasites. Interestingly, results obtained from invasion assays showed that parasite exhibited a protease release higher than that found among the parasites collected from interaction assays. Furthermore, by using video microscopy we followed the parasite behavior during migration in 3D and 2D. Under interaction with COL at 2D, most of parasites presented a migration rate higher than that observed among those at 3D. Altogether, the results here presented demonstrated that the supramolecular structure of the environment where *E. histolytica* is found greatly influence each one of morphology, adhesion, migration rates, and protease activity of the parasite. Supported by CNPq, FAPERJ, FAPEMIG, and INPeTAm.

Keywords: collagen, 3D matrix, *entamoeba histolytica*

05.7- Early Events Related to the Interaction between Leishmania Amazonensis and 3D Collagen-I Matrix

Débora Petrópolis, Kassia Ignacio, Juliany Cola Rodrigues, Fernando Costa Silva-Filho

1. UFRJ, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Leishmaniasis is a widely world spread disease in which *Leishmania leishmania amazonensis* is the causative agent of the related cutaneous form. Recent published data also demonstrated that the parasite alters the extracellular matrix (ECM) organization on the infection site on mice footpad. The ECM has indeed a key role in the pathogenesis associated to *Leishmania*. It seems to us that ECM may determine parasites preferred niche as well as to serve as molecular roadmaps into hosts. Data available on the interaction between parasitic protozoa and ECM underlie the existence of a bidirectional signal exchanging from ECM to parasites and from parasites to ECM. Both chemistry and mechanics concerning networks

formed by ECM may trigger the activation of cell and tissue invasiveness by parasites. The present study explores the mechanochemical interaction between *L. amazonensis* promastigotes and each one of 2D and 3D matrices, all made of Collagen-I (COL). Promastigote forms of *L. amazonensis* were here allowed to interact with each one of glass slides, 50 µg ml⁻¹ COL-coated glass slides (2D), and 3.0 mg.ml⁻¹ COL (immobilized or unattached both at 3D) which in turn, was leaded to polymerize for 1 h at 37°C. In order to follow the ability of the parasite to remodel COL matrix it was performed an contraction assay were COL gel were unattached from the bottom of the plastic surface. The resulting alterations of the total unattached COL gel area were analysed by digital scanning of the gels and resulting images were analyzed by using the Image Pro Plus 4.0 software. The total area of the 3D COL unattached gel was 1.2 times smaller after 24h of interaction with promastigotes. Following such interactive process at light microscopy we found that most promastigotes remained migratory even when associated to COL at 3D. Otherwise, after 24h of *Leishmania*-COL interaction many parasites seemed to scape from the both forms of 3D COL matrix (immobilized and unattached). It seems that promastigotes forms can process the migration habituity on their host tissue and that migration could start an remodelling process of the ECM. SDS-PAGE analysis was carried out in order to investigate the proteins released by the parasites during their cultivation in each one of 2D and 3D environments. Interestingly, three different bands of high proteins mass could only be observed on samples from *L. amazonensis* cultivated on 3D COL matrices. Supported by CNPq and INPeTAm.

Keywords: *Collagen I, Leishmania, 3D matrix*

05.8- A Protein Analysis of the Protozoan *Acanthamoeba Polyphaga* before and After its Binding to Immobilized Laminin 1

*Kassia Leone Ignacio¹, Débora Barreiros Petrópolis¹, Bruno da Rocha Azevedo²,
Fernando Costa e Silva Filho¹*

1. UFRJ, Universidade Federal do Rio de Janeiro, Bl G do CCS, sala G0-44, Cidade Universitária, Ilha do Fundão-CEP:21941-902. 2. VCU, Virginia Commonwealth University, Richmond, VA, USA

Acanthamoeba polyphaga is a free-living amoebae that cause human opportunistic infections such as granulomatous encephalitis, osteomyelitis and keratitis. The last which has been mostly observed among contact lens users seems to be initiated by the binding of *A. polyphaga* trophozoites to Laminin 1 (LMN). In in vitro conditions such trophozoites are also able to recognize others extracellular matrix (ECM) components including fibronectin, collagen I and elastin, all in a cation-dependent manner. Otherwise, previous contact of amoeba trophozoites to ECM molecules seems to become the protozoan highly adherent and toxic to mammalian cultured cells. Thus, it seems to us that the *A. polyphaga*-ECM interaction may play important roles in the human host tissues invasion exhibited by the protozoan. The LMN recognition by *A. polyphaga* has been reported to result in both morphological and physiological alterations in the protozoan. Here, we investigated the binding of *A. polyphaga* trophozoites to immobilized LMN by focusing the proteins released by the protozoan during its interaction with this ECM macromolecule. For the experiments,

twenty-four-multiwell plates were incubated for two hours at 37°C with 20 µg/ml LMN-I in two different pHs (4.4 and 6.6). After coating, wells were washed gently once with PBS in order to remove unadsorbed LMN. Trophozoites grown in PYG medium at 25°C were detached and washed twice in PBS, and suspended in a buffer containing divalent cations (Ca^{+2} , Mg^{+2} , Mn^{+2}). This suspension was led to interact with each one of uncoated or LMN-coated surfaces (400000 trophozoites/cm²) for until 120 min, at 25°C. Following the incubation, the supernatants were collected and the secreted proteins profiling was revealed by SDS-PAGE using silver staining. Protein profiles showed in SDS-PAGE revealed that among the bands observed, two bands of medium molecular weight (between the 60 and 35 kDa markers) were only detected in the presence of LMN-I. However, any remarkable difference was not found in terms of protein bands when amoebae interacted for 20 min on LMN. Collectively, our data suggest that trophozoite binding to LMN-I possibly induce modifications on the amoeba cell signaling, leading to the release of differential proteins, in a time-dependent manner. Supported by PIBIC (to KLI), CNPq (scholarship to DBP), and INPeTAm-Instituto Nacional de Pesquisa Translacional Integrando Saúde e Ambiente na Região Amazônica.

Keywords: *Acanthamoeba*, *Laminin*, *SDS-PAGE*, *Cell signaling*, *Silver staining*

06. ECM AND PATHOLOGICAL PROCESSES

06.1- Anti-angiogenic effect of a Heparin-Like Glycosaminoglycan Isolated From a Marine Shrimp

Juliana L. Dreyfuss¹, Caio V. Regatieri^{1,2}, Marcelo A. Lima^{1,3}, Eduardo H. Farias¹, Adriana S. Brito³, Suely F. Chavante³, Helena Bonciani Nader¹

1. UNIFESP, Universidade Federal de São Paulo, Molecular Biology Division, Rua Tres de Maio, 100, São Paulo, Brazil. 2. UNIFESP, Universidade Federal de São Paulo, Ophthalmology Department. Rua Botucatu, 820, São Paulo, Brazil. 3. UFRN, Universidade Federal do Rio Grande do Norte, Biochemistry Department. Campus Universitario, Natal, Brazil

It has been shown that a heparin-like glycosaminoglycan obtained from shrimp cephalothorax (*Litopenaeus vannamei*) is capable to modulate inflammatory responses without interfering on hemostasis. Besides it reduced bleeding potential and anticoagulant activity in vitro, the shrimp heparin-like compound is able to reduce the activity of matrix metalloproteinases. Thus the objective of this study was to evaluate the anti-angiogenic effects of this new compound on an “in vitro” and “in vivo” models. The ability of endothelial cells form capillary-like structures “in vitro” when plated on top of a reconstituted basement membrane extracellular matrix (Matrigel) was investigated in cells treated with the heparin-like compound (0.09, 0.9 and 9 ig/mL). The cytotoxicity of this compound was evaluated by MTT test in retinal pigmented epithelial cells (ARPE-19) and endothelial cell cultures. The “in vivo” model to evaluate the anti-angiogenic effect of this compound was the laser-induced choroidal neovascularization (CNV) in rats. The CNV was induced using argon

laser, and at the end of laser session, saline or heparin-like glycosaminoglycan were injected intravitreally in the 4 groups of animals (laser, and laser with 0.09, 0.9 and 9 $\mu\text{g/mL}$ of heparin-like glycosaminoglycan). After three weeks, the eyes were enucleated and immunofluorescence was performed (flatmount) using anti-von Willebrand factor, a marker for endothelial cells. All three tested doses of the heparin-like glycosaminoglycan induced a significant decrease ($p < 0.05$) in total length of tubes formed by endothelial cells in Matrigel. Immunofluorescence analysis showed significant reduction in CNV lesion area of all groups treated with heparin-like glycosaminoglycan. The regression observed was 28%, 53% and 41% in 0.09, 0.9 and 9 $\mu\text{g/mL}$ treated groups, respectively ($P < 0.0001$). No cytotoxic effect was detected in ARPE-19 or endothelial cell culture. Intravitreal injection of the the heparin-like glycosaminoglycan from a marine shrimp significantly reduces the CNV lesion area. The optimal dosage to reduce angiogenesis was 0.9 $\mu\text{g/mL}$ and it was no cytotoxic effect in ARPE-19 cells or endothelial cells. These results demonstrated a new useful antiangiogenic compound that can be used as a therapy to control neovascularization. Supported by FAPESP, CNPq and CAPES.

Keywords: *extracellular matrix, inflammation, glycosaminoglycans, neovascularization*

06.2- Gene Expression Profile of Matrix Metalloproteinases and their Inhibitors in Human Cholesteatomas

*Laís de Campos, Carlos Eduardo Rezende, Priscila Bogar Rapoport,
Ricardo Peres do Souto*

1. FMABC, Faculdade de Medicina do ABC, Av. Lauro Gomes, 2000 - Santo André, SP
- 09060-870

Cholesteatoma is a middle-ear cyst formed by epidermal keratinized tissue with the capacity of migration and erosion of adjacent structures. Cholesteatoma may compromise the ossicular chain, favor middle ear infections and cause irreversible damage, leading to permanent hearing disability. The matrix metalloproteinases (MMPs) are enzymes involved in degradation of extracellular matrix (ECM) and are regulated by specific tissue inhibitors (TIMPs). The balance between MMPs and TIMPs is critical to determine the integrity and function of ECM, and thus variations in the presence and activity of these proteins may contribute to many of the tissue events observed in cholesteatoma. The purpose of this study is to determine the gene expression profiles of MMP and TIMP isoforms in human cholesteatomas and to evaluate the influence of such profile to the aggressiveness of the disease. For analysis of gene expression, samples from patients with clinical and radiological diagnosis of chronic otitis media with cholesteatoma were surgically removed, immediately frozen in liquid nitrogen and stored at -70°C . Total RNA extraction was carried out by guanidinium extraction and ethanol precipitation or silica column affinity. Amplification of MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1 and the positive control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by RT-PCR. Eleven cholesteatomas were processed, all expressed GAPDH. Preliminary analysis of six samples showed expression of MMP-2, MMP-3 and TIMP-1 in one case, and MMP-2, MMP-13 and TIMP-1 in another sample. The other four cholesteatomas did not express any of these genes. MMP-9 expression

was negative for all six samples. The presence of MMP-2, MMP-9, MMP-13 and MMP-3 in cholesteatoma has been reported previously in the literature respectively by immunohistochemistry and zymogram. This is the first time that TIMP-1 expression is observed in cholesteatoma. Preliminary results showed different expression of MMPs and TIMPs in cholesteatomas, so this analysis could have value for classification of lesions and prognosis. Financial Support: PIBIC/CNPq and NEPAS

Keywords: *Cholesteatomas, Gene expression, Matrix metalloproteinases, RT-PCR*

06.3- MAGP-1 and Thrombus Formation

Denise Machado¹, Camila Kopecky Possari¹, Tallita Vassequi da Silva¹, Cristina Pontes Vicente², Thomas John Broekelmann³, Robert Paul Mecham³, Claudio Chrysostomo Werneck²

1. UNICAMP, State University of Campinas, R Monteiro Lobato s/n CP6109 Dep. Bioquímica- Inst.Biologia CEP13083970. 2. UNICAMP, State University of Campinas, R Monteiro Lobato s/n CP6109 Dep. ABCF- I.Biologia CEP13083970. 3. WUSM, Washington University School of Medicine, 660 S. Euclid Ave, Saint Louis, MO 63110 Box 8228

Mice lacking the extracellular matrix protein Microfibril Associated Glycoprotein-1 (MAGP1) display delayed thrombotic occlusion of the carotid artery following injury by a photochemical assay as well as prolonged bleeding time from a tail vein incision. Normal occlusion times were restored when recombinant MAGP1 was infused into deficient animals prior to vessel wounding. Blood coagulation was normal in these animals as assessed by activated partial thromboplastin time (aPTT) and prothrombin time (PT). Platelet number was lower in MAGP1-deficient mice, but the platelets showed normal aggregation properties in response to various agonists. MAGP1 was not found in normal platelets or in the plasma wild-type mice. Looking for a possible partner for MAGP1 by ligand blot assays, MAGP1 binds to fibronectin, fibrinogen, and von Willebrand factor, but von Willebrand factor was the only protein of the three that binds to MAGP1 in surface plasmon resonance studies. Mapping MAGP1 domains important for occlusion time rescue suggest that RYVVVNK peptide is playing a role in that. Taking into consideration that TGF-beta is released from platelets during thrombus formation we decided investigate if Losartan, a putative antagonist of angiotensin II receptor AT1, that decreases TGF-beta expression and activation, has any effect in this process. MAGP1 deficient mice Losartan treated had their occlusion time decreased to wild type mice levels. Treatments used were effective in recovering the normal occlusion time in Magp1-ko mice suggesting that TGF-beta, may be a important player in this model of hemostasis. Financial support: FAPESP, CNPq and CAPES.

Keywords: *thrombosis, elastic fiber, MAGP1, microfibril*

06.4- Involvement of TGF-Beta and VEGF in Bone Regeneration in Critical Defects in Rats Calvaria

Roosevelt K Florim², Rodrigo Florim², Gustavo Fernandes de Oliveira³, Tadeu Lima Montagnoli¹, Carmelo Sansone², Radovan Borojevic¹, José Mauro Granjeiro¹, Christina Maeda Takiya¹

1. UFRJ, Universidade Federal do Rio de Janeiro- ICB, Av Carlos Chagas 373 Ilha do Fundão. 2. UFRJ, Universidade Federal do Rio de Janeiro Fac.Odontologia, Av Carlos Chagas 373 Ilha do Fundão. 3. UFF, Universidade Federal Fluminense Instituto de Biologia, Outeiro de São João Batista

Introduction: Periodontal bone defects are highly prevalent in adults worldwide and are still a challenge in dental regeneration procedures. Therefore the use of biomaterials and growth factors are already clinically employed, however further researches are required to better fulfillment in critical bone defects. **Objectives:** In this study, we evaluated bone regeneration with different biomaterials in critical bone defects by histological and histomorphometrical techniques associated to immunohistochemical localization of TGF-beta, VEGF, and macrophages. **Methodology:** Calvaria bone defects (5 mm) were performed in Wistar rats fulfilled with autogenous bone, bioglass, hidroxyapatite, and bioOss. All animals were sacrificed after 15 and 45 days post surgery and calvarias were fixed in 4% buffered formaldehyde, and decalcificated in Morse solution. Paraffin sections were stained with hematoxylin-eosine, Masson's trichrome, and picro Sirius red. For Immunohistochemistry, antibodies against rat ED1 antigen, , and VEGF were applied on paraffin sections and revealed with the β pan TGF- LSAB®-HRP kit for use in rat tissue specimens (DAKO) and the chromogen was diaminobenzidine. **Results:** The histological analysis revealed that after 45 days new bone formation almost fulfilled the bottom of the defects, mainly with autogenous and bioOss groups. Morphometrically a significant amount of neoformed bone was only achieved in animals that received autogenous bone after 45 days ($p < 0.05$) while diminution of connective tissue formation was statistically significant after 45 days in autogenous bone group. Different subpopulations of ED1+ macrophages were present in the different groups. In neoformed bone, periosteal and endosteal surfaces were partially covered with ED1+ cells. TGF-beta reactivity was present in inflammatory cells, mainly macrophages and in osteoblasts, aspect predominant in all 15 days groups, diminishing in the 45 days groups. VEGF also was present in inflammatory cells and mesenchymal osteoprogenitors and osteoblastic cells covering periosteal and endosteal surfaces both at 15 and 45 days after surgery. **Conclusions:** It seems that regeneration of calvaria bone defects requires the presence of various macrophagic population and that both TGF-beta and VEGF are involved in the signal transduction leading to osteoprogenitor/osteoblast activation

Keywords: bone defect, regeneration, TGF beta, VEGF

06.5- Expression of Decorin and Biglycan in Human Normal Term Placenta and in Invasiveness-Changed Trophoblast Pathologies

Alexandre Urban Borbely¹, Márcia Marcelino Ishigai², Silvia Daher², Rosiane Mattar², Sue Yazaki Sun², Estela Bevilacqua¹, Sérgio Ferreira de Oliveira¹

1. ICB/USP, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1524. 2. EPM/UNIFESP, Escola Paulista Medicina, Universidade Federal de São Paulo, R. Botucatu, 862

Decorin and biglycan are members of the small leucine-rich proteoglycans (SLRPs) family, and are constituents of both extracellular matrix and cell surface. In the extracellular matrix, decorin and biglycan have been pointed as important factors in the control of trophoblast proliferation, migration and invasion. There are some pathologies that present an increased invasive activity of the trophoblast. These pathologies can be developed during or after gestation, and could be fatal for both mother and fetus, like the placenta accreta, invasive mole and choriocarcinoma. The aim of this study was to characterize, throughout immunohistochemistry analyses, the differential expression and the localization of proteoglycans, decorin and biglycan, in normal term placenta, placenta accreta, invasive mole and choriocarcinoma. In normal term placenta, decidual cells presented positivity for decorin whereas the extravillous cytotrophoblast was negative. Decorin was weakly stained in endometrial matrix, however, the matrix-type fibrinoid was not reactive. In placenta accreta and invasive mole, extravillous cytotrophoblast cells were positive for decorin whereas endometrial matrix was negative. In choriocarcinoma samples, only cytotrophoblast cells and metastatic cells were immunoreactive for decorin, including mitotic figures of cytotrophoblast cells. Immunohistochemistry for biglycan showed similar results to decorin immunohistochemistry in almost all cases, with exception of the matrix-type fibrinoid in normal term placenta and placenta accreta that presented strong staining for biglycan. These results demonstrated that decorin and biglycan are differentially expressed in normal term placenta and in placental pathologies. These results further suggest that the expression patterns of both proteoglycans in placenta accreta, invasive mole and choriocarcinoma might play a possible role in modulating trophoblast migration and invasion.

Keywords: *Biglycan, Decorin, Human Term Placenta, Placenta Pathologies, Trophoblast Invasion*

06.6- High Affinity Binding with Collagen Determines the Vascular Damage Induced by Hemorrhagic Snake Venom Metalloproteinases

Cristiani Baldo¹, Norma Yamanouye², Colin Jamora³, Telma Maria Tenório Zorn⁴, Ana Maria Moura da Silva¹

1. Instituto Butantan, Laboratório de Imunopatologia, São Paulo, SP. 2. Instituto Butantan, Laboratório de Farmacologia, São Paulo, SP. 3. UCSD, Section of Cell and Developmental Biology, San Diego, CA, USA. 4. ICB - USP, Laboratório da Biologia da Reprodução e Matriz Extracelular, São Paulo, SP

Local hemorrhage is an important symptom of viper venom envenoming correlated to the action of Snake Venom Metalloproteinases (SVMPs). This action depends on SVMPs catalytic action on basement membrane components. However, although all SVMPs are catalytic, only class P-III SVMPs induce strong hemorrhage while P-I SVMPs exhibit lower activity. This evidence suggests that hemorrhage is modulated by adhesive mechanisms involving the non-catalytic domains, present only in P-III SVMPs. In order to test this hypothesis, we compared hemorrhagic lesions induced by jararhagin (highly hemorrhagic P-III) and BnP1 (weakly hemorrhagic P-I) using the mouse skin as experimental model. As previously reported, 15 minutes after injection of equimolar doses of toxins, only jararhagin induced strong skin hemorrhage, located mainly in hypodermis. Tissue localization of Alexa488-labeled toxins revealed an accumulation of jararhagin around venules and capillary vessels labeled by anti CD-31, and a co-localization with basement membrane collagen IV. The same distribution pattern was detected with jararhagin-C, which has only disintegrin-like and cysteine-rich domains of jararhagin. In contrast, BnP1 did not accumulate in the vicinity of blood vessels and did not co-localize with collagen IV. After tissue accumulation of jararhagin, a strong disorganization and degradation of collagen fibers was observed in hypodermis stained by picrossirius-hematoxilin. Also, a marked degradation of collagen IV in the basement membrane was observed by immunofluorescence using anti-collagen IV. Similar effects were not detected using BnP1. Thus, we suggest that binding to collagen IV through the non-catalytic domains leads to the accumulation of hemorrhagic SVMPs on blood vessel walls, and consequent damage of its basement membrane. This effect causes weakening of the capillary vessel and results in the strong local hemorrhage induced by P-III SVMPs. Supported by INCTTOX Program, FAPESP and CNPq

Keywords: *collagen, metalloproteinases, basement membrabe, hemorrhage, snake venom*

06.7- A Selective Cyclooxygenase-2 Inhibitor Suppresses the Growth of Endometriosis with an Anti-Angiogenic Effect in a Rat Model

Daniel Escorsim Machado¹, Plínio Tostes Berardo¹, Richardt Gama Landgraf², Patrícia Dias Fernandes¹, Célia Palmero¹, Leandro Miranda Alves¹, Maurício Simões Abrao², Luiz Eurico Nasciutti¹

1. UFRJ, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde. 2. USP, Universidade de São Paulo, Faculdade de Medicina

Endometriosis is believed to be the result of implantation of retrograde shed endometrium during menstruation, causing chronic pelvic pain, dysmenorrhea and infertility. The survival of ectopic endometrium is related to the acquisition of an adequate blood supply, and the use of angiostatic agents promises a new therapeutic option. Cyclooxygenase-2, a rate-limiting enzyme in the biosynthesis of prostaglandin E2 (PGE2), is highly expressed in the endometriotic tissue and results in an increase of the peritoneal PGE2 concentration. Although the inhibition of COX-2 prevents the establishment and growth of endometriosis lesions in different animal models, the mechanisms underlying this regression remain to be further determined. In this study, we investigated the antiangiogenic effects of the selective COX-2 inhibitor Parecoxib on the growth of endometrial implants in a peritoneal

endometriosis rat model. We analyzed the vascular density, the expression of vascular endothelial growth factor (VEGF) and its receptor Flk-1, the distribution of activated macrophages, the expression of COX-2 and the prostaglandin concentration in the endometriotic lesions treated with Parecoxib for 30 days. The treatment significantly decreased the implant size, and the histological examination indicated mostly atrophy and regression. It was also observed a reduction in the microvessel density and in the number of macrophages, associated to a decrease of VEGF and FLK-1 expression. Finally, the treatment group showed low concentration of PGE2. These results suggest that the use of COX-2 selective inhibitors could be effective to suppress the establishment and growth of endometriosis, partially by their antiangiogenic activity. Studies of this agent in humans as a potential therapy are indicated.

Keywords: *Angiogenesis, Cyclooxygenase-2 inhibitor, Endometriosis, Prostaglandin, Vascular Endothelial Growth Factor*

06.8- Cathepsin B Activity as a Viral Infection Marker in Asymptomatic Blood Donors

*Camila Melo Accardo¹, Madalena Maria Nunes da Silva Pares³, José Augusto Barreto³,
Helena B. Nader¹, Ivarne Luis Santos Tersariol^{2,1}*

1. UNIFESP, Universidade Federal de São Paulo, R. Três de Maio, 100 - 4º andar - VI. Clementino CEP 04044-020. 2. UMC, Universidade de Mogi das Cruzes, Av. Dr. Cândido Xavier de Almeida Souza, 200-Prédio I - C. Cívico CEP 08701-970. 3. COLSAN/UNIFESP, Associação Beneficente de Coleta de Sangue, Av. Jandira, 1.260 - Indianópolis CEP 04080-006

Cathepsin B is a lysosomal cysteine protease widely distributed in tissues that can degrade extracellular matrix (ECM) molecules. This enzyme that can be released in serum is involved in various physiological roles like antigen presentation, apoptosis and inflammatory processes, and in pathological events as tumor progression, metastasis and viral infections. This work suggests the utilization of cathepsin B level as marker of viral infection in asymptomatic blood donors. Two hundred three serum samples of healthy blood donors (control group, CT) and forty one serum samples of blood donors that had blood bag excluded after donation because serological viral positive results (SVPR) to: HIV (N=5); HTLV (N=5); HCV (N=16) and HBV (N=15) have the cathepsin B activity measured spectrofluorometrically, using the fluorogenic substrate Z-FR-MCA at 37°C in 50 mM sodium phosphate buffer, pH 6.3, containing 200 mM NaCl and 2 mM EDTA. Fluorescence intensity was monitored in a microplate reader, with excitation and emission wavelengths set as 365 and 420 nm, respectively. The assay was performed by preincubating the serum samples with the enzyme activator 2 mM DTT for 20 minutes and then adding the irreversible inhibitor E-64. Cathepsin B data are expressed in UAF/μg protein as mean ± SE. Statistical analysis performed by ANOVA and Receiver Operating Characteristic (ROC) curve tests. Cathepsin B level in CT group was 24.97 ± 4.08 while in SVPR groups it was observed a remarkable increase: HIV - 873.7 ± 497.8 ; HTLV - $1,850.0 \pm 987$; HCV - 838.6 ± 228.6 ; HBV - 338.9 ± 91.1 . Statistical studies of cathepsin B diagnostic properties, show sensibility

96.2%, specificity 91% and cut points between healthy, serological positive retroviruses (HIV and HTLV) and other viruses (HCV and HBV). The results suggests cathepsin B as a new diagnostic marker for viral infections, being possible to establish different values to act like biomarker for different virus type, cathepsin B showed to be a stable marker without epidemiology interferences. (Supported by CAPES, CNPq and FAPESP).

Keywords: *Blood Donors, Cathepsin B, Diagnostic Marker, Viral Infections*

06.9- Extracellular Matrix and its Relationship With Palatopharyngeal Muscle in the Sleep Apnea Syndrome

Sebastião Roberto Taboga¹, Fernanda Cristina Alcantara Santos¹, Luis Roberto Falleiros Jr¹, Eny Maria Goloni-Bertolo², Sergio Luis Felisbino³, Luis Antonio Justulin-Jr³, José Victor Maniglia², Fernando Drimel Molina²

1. IBILCE, Instituto de Biociências, Letras e Ciências Exatas - UNESP, R Cristovão Colombo, 2265 - São José do Rio Preto, 15054000, SP, Brasil. 2. FAMERP, School of Medicine of São José do Rio Preto, São José do Rio Preto, SP, Brasil. 3. IB, Instituto de Biociências - UNESP, Distrito de Rubião Júnior, Botucatu, SP, Brasil

Obstructive Sleep Apnea Syndrome (OSAS) is a complex disease characterized by continuous and intermittent obstructive events of the airways. This can evolve into systemic complications of great importance for the patient's metabolism and quality of life. OSAS physiopathology is multifactor condition, being the airways compliance a very important component. The aim of this study was to evaluate the tissue changes in the palatopharyngeal muscle by morphometric-stereological and immunohistochemical analysis, with special attention to extracellular matrix components associated with this muscle at structural and ultrastructural levels. Thirty patients with OSAS were divided in groups of 10 according to disease severity: mild (Mi), moderate (Md) and serious (S) OSAS. In addition, the control group (Ct) consisted of 10 patients. Fragments of palatopharyngeal muscle removed from patients with OSAS, and tonsillectomies from patients in the Ct group were submitted to light and transmission electron microscopy. The results showed important differences in analyzed groups, such as reduction of the muscle fibers diameter in patients with OSAS (mayor diameter = Ct: $112.4 \pm 30.9 \mu\text{m}$, Mi-OSAS: $80.1 \pm 16.9 \mu\text{m}$, Md-OSAS: $80.7 \pm 13.3 \mu\text{m}$, S-OSAS: $81.6 \pm 17.1 \mu\text{m}$). The stereological data showed a progressive increase in the relative frequency of collagen and elastic fibers, when comparing the patients in the Ct group (collagen: $11.3 \pm 3.6\%$; elastic fiber: $9 \pm 2.8\%$) those patients with OSAS (collagen = Mi-OSAS: $16.5 \pm 4\%$, Md-OSAS: $14.9 \pm 3.2\%$, S-OSAS: $19.4 \pm 6.4\%$; elastic fibers = Mi-OSAS: $15.3 \pm 4\%$, Md-OSAS: $14.9 \pm 3.5\%$, S-OSAS: $16.6 \pm 3.3\%$; $p \leq 0.05$). The immunostaining for MMP-2 and MMP-9 demonstrated weak reaction in the connective tissue and in the muscle fibers cytoplasm from Ct patients. These enzymes were increased in both muscle fibers cytoplasm and connective tissue in the samples from the patients with different levels of OSAS. The ultrastructural analysis showed that palatopharyngeal muscle fibers of OSAS group presented early cellular aging, characterized by the presence of autophagic vesicles and heterogeneous residual bodies. We can conclude that the increase of tissue compliance in individuals with OSAS may be, among other factors, a consequence of the

muscle fiber contractile reduction, which exhibited clear signs of early senescence. Moreover, extracellular matrix components changes may contribute to the muscle myopathy during OSAS progression.

Keywords: *colagen fibers, elastic system, MMPs, sleep apnea syndrome, palatopharyngeal mucle*

06.10- Composition of Sulfated Glycosaminoglycans and Immunodistribution of Chondroitin Sulfate in Deeply Infiltrating Endometriosis Affecting the Rectosigmoid

Plínio Tostes Berardo¹, Renata Azevedo da Costa¹, Maurício Simões Abrao², Maísa Souza³, Daniel Escorsim Machado¹, Luiz Cláudio Silva¹, Luiz Eurico Nasciutti¹

1. UFRJ, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde. 2. USP, Universidade de São Paulo, Faculdade de Medicina. 3. CEFETQ, Centro Federal de Educação Tecnológica de Química, CEFETQ - Nilópolis

Endometriosis is a prevalent condition in which endometrium-like glandular and stromal tissue exists at ectopic sites outside the uterus. Ectopic lesions exhibit recurrent menstruation-like bleeding that result in blood-filled cysts, local inflammatory reactions and fibrosis. Endometriosis affects approximately 10% of the female population in their reproductive years, and women with endometriosis present characteristic signs and symptoms: dysmenorrhea, dyspareunia, chronic pelvic pain or sub-fertility. Deeply infiltrating endometriosis is defined as pelvic endometriosis infiltrating deeper than 5 mm below the peritoneum, and the major component of the lesions is fibromuscular tissue. Bowel endometriosis is one of the greatest concerns of specialists treating deep infiltrative endometriosis because of the severity of its symptoms, concomitant infiltration of pelvic organs, the possibility of bowel obstruction resulting from the progression of the disease, and the technical difficulties in surgical removal. In the present study, we investigated the composition of sulfated GAGs and the tissue distribution of chondroitin sulfate (CS) in deeply infiltrating endometriosis (DIE), using histochemical, biochemical and immunohistochemical analyses. The sulfated GAGs were characterized as dermatan sulfate (DS), heparan sulfate (HS) and CS; and DS strongly predominated compared to HS and CS. Immunostaining procedures showed that CS was concentrated in the endometriosis foci, distributed throughout the stroma around the glands. This is the first report describing the composition of sulfated GAGs and the tissue location of CS in DIE by means of histochemical, biochemical and immunohistochemical analyses. These results confirmed that in DIE of rectosigmoid, as in eutopic endometrium, CS was the dominant sulfated GAG in stroma of the lesion foci.

Keywords: *Chondroitin sulfate, Deeply infiltrating endometriosis, Glycosaminoglycan, Human endometrium*

06.11- Extracellular Matrix Changes of Cartilage and Sinovial Tissue in Diabetic Rats

Sandra Aparecida Atayde, Dafne Port Nascimento, Sérgio Catanozi, Vera Luiza Capelozzi, Natalino Hajime Yoshinari, Walcy Rosolia Teodoro

1. FMUSP, Faculdade de Medicina da Universidade de Sao Paulo, Avenida Doutor Arnaldo, 455 3andar sala 3024

Background: Changes in extracellular matrix components of tendons and ligaments are frequently observed in diabetic patients. It can result in angiogenesis, healing, inflammatory response and cells proliferation delays, what may be involved in limited joint mobility. The cartilage and synovial tissue disease can have be relationship with limited joint mobility in diabetic patients. **Purpose:** To evaluate the collagen and proteoglycan cartilage amount and synovial tissue architerture after the development of diabetes by streptozotocin inoculation in rats. **Methods:** We used a 10 weeks-old Wistar rats (N=30), weighting 200-250g divided into two groups: diabetic group (DG=15) and control group (CG=15). Diabetes was induced by streptozotocin caudal injection (35mg/kg) and the CG received physiological solution. The animals were monitored to weight and blood glucose during 10 weeks. The femorotibial joints were isolated, immersed in formalin 10%, decalcificated, included in paraffin and stained with Hematoxilin-Eosin (HandE), Picrosírius and Safranin-0 with "Fast-green". The quantitative analysis of cartilage collagen and proteoglycan was evaluated in Olympus BX-51 microscopy by software Image Pro-Plus 6.0 in different randomly selected fields using Picrosírius and Safranin-0. The synovial tissue histoarchiterture was evaluated in HandE, Masson's tricomc and Picrosírius. The collagen II and XI cartilage expression was evaluated by immunofluorescence. **Results:** It was found higher blood glucose levels in the DG when compared to CG (426.2 ± 65.4 vs 97.46 ± 6.7 ml/dl; $p < 0.01$). The weight of DG was significantly lower than in CG (263 ± 19.97 vs 460.46 ± 65.43 g; $p < 0.01$). The morphometric evaluation showed an collagen thin fibers increase ($4,79 \pm 7,62$ vs $23,06 \pm 16,07$; $p = 0,01$), an decrease of thick fibers collagen ($33,05 \pm 8,53$ vs $28,64 \pm 8,21$; $p = 0,04$) and proteoglycan expression ($343,53 \pm 156,66$ vs $186,07 \pm 146,35$ $p = 0,03$) in DG. Was observed collagen XI deposition in DG cartilage. In addiction the synovial analysis by HandE showed inflammatory infiltrate absence and the morphological analysis of collagen shows adipose tissue substitution for fibrous tissue thickness collagen fibers in DG. **Conclusions:** This cartilage and synovial remodeling can be responsible for articular dysfunction in diabetic rats, suggesting that this process is the result of pathophysiological mechanisms in this illness.

Keywords: *joint, experimental diabetes, cartilage, collagen, remodeling*

06.12- Fibrinolytic Activities are Associated with Smooth Muscle Cells in Aneurysms of the Ascending Aorta

Luciano F Borges^{1,2}, Delphine Gomez¹, Mercedes Quintana¹, Anne Leclercq¹, Ziad Touat¹, Guillaume Jondeau¹, Olivier Meilhac¹, Martine Jandrot-Perrus¹, Paulo Sampaio Gutierrez³, Edna Freymuller², Roger Vranckx¹, Jean-Baptiste Michel¹

¹ Inserm, U698, CHU X. Bichat, Paris; University of Paris 7 (Denis Diderot), France;

²Electron Microscopy Center, University Federal of São Paulo, Brazil; ³ Laboratory of Pathology, Heart Institute (InCor), School of Medicine, University of São Paulo, Brazil

Background: Human aneurysms of the thoracic ascending aorta (TAA) are characterized histologically by fibrillar extracellular matrix breakdown and mucoid degeneration within the aortic media associated with progressive smooth muscle cell (SMC) rarefaction. The TGF- β 1/Smad2 signaling pathway is assumed to play a role in this process. Since the fibrinolytic system is able to activate MMPs, to induce SMC disappearance, and to increase the bio-availability of TGF- β 1, we explored the plasminergic system within the aortic wall in TAA. **Material and Methods:** Ascending aortas from 19 controls and 23 patients with TAA were analyzed by immunohistochemical, biochemical and molecular approaches. **Results:** Immunohistochemical approaches showed that the serine-proteases (tPA, uPA and plasmin) accumulated in close association with the remaining SMCs in the TAA media but were absent from the mucoid material. The over-expression of t-PA, and u-PA in the lesions was confirmed at the transcript level by RT-PCR and at the protein level by immunoblotting on tissue extracts and tissue-conditioned media. Plasminogen was also associated with the remaining SMCs, at their surface and inside cytoplasmic vesicles, but plasminogen synthesis (mRNA) was undetectable in the aortic media. Activation of serine proteases was assessed by zymography, showing low molecular weight u-PA and by immunoblotting for plasmin and proteases-antiproteases complexes. Plasmin-antiplasmin complexes were detected in tissue-conditioned media. Activation of the fibrinolytic system was associated with an increased production of fibronectin-degradation products released in tissue-conditioned media. Within tissue, fibronectin-related material appeared in dense clumps around SMCs and co-localized with latent TGF- β Binding Protein-1 (LTBP-1). **Conclusion:** Together, these observations indicate that serine proteases of the fibrinolytic pathway could play a critical role in the progression of TAA, via proteolysis of extracellular matrix molecules and consecutive modulation of TGF- β 1 bio-availability.

07. ECM AND DEVELOPMENT

07.1- Differential Expression of Heparanase During Neonatal Ventral Prostate Development in Wistar Rats

Guilherme Oliveira Barbosa, Taize Machado Augusto, Alexandre Bruni-Cardoso, Hernandes Faustino de Carvalho

1. UNICAMP, State University of Campinas, Campinas SP, Brazil

There are a number of enzymes involved in the development of ventral prostate (VP) in the first week after birth. Matrix Metalloproteinase-2 and 9 have been implicated in extracellular matrix (ECM) remodeling and signaling by degrading collagen, proteoglycans, and other components of ECM and allowing epithelial growth towards the stroma (Bruni-Cardoso et al., *Histochem Cell Biol* 129:805, 2008). Heparanase is an endoglycosidase that degrades heparan sulfate, an important component of the basal membrane, which acts as a

reservoir of growth factors regulating different aspects of cell physiology. It has been reported that this enzyme is relevant in tumor invasiveness and metastasis (Ilan et al., *Int J Biochem Cell Biol* 38: 2018-2039, 2006), angiogenesis, development (Patel et al., *Development* 134, 4177-4186, 2007), and also ECM remodeling in adult VP after castration (Augusto et al., *Cell Tissue Res* 332:307, 2008). We hypothesized that heparanase-1 activity would have a relevant role in coordinating the dynamics of the epithelium and stromal remodeling during the early postnatal development of the rat VP, and used Western blotting, RT-PCR and qRT-PCR to compare heparanase-1 protein and mRNA content at days 0 (day of birth), 3, 6, and 91. Heparanase was found during VP development and the protein level increased after birth, reaching a plateau after day 3. The amount of the pro-enzyme showed a similar behavior. Activation (the ratio between the active form and the pro-enzyme) was constant during this period. The level of heparanase-1 mRNA also increased from day 0 to day 6. These results showed that heparanase is present during VP development and may play an important role in ECM remodeling and cell signaling by degrading heparan sulfate proteoglycans and releasing bioactive molecules.

Keywords: *Heparanase, Developmet, Ventral prostate, Neonatal*

07.2- MMP-2 Modulates the Development of the Rodent Ventral Prostate

Alexandre Bruni Cardoso, Rafaela da Rosa Ribeiro, Hernandes Faustino de Carvalho
1. UNICAMP, Universidade Estadual de Campinas, Cidade Universitária "Zeferino Vaz"

We have shown before that matrix metalloproteinases (MMP) exhibit localized distribution during the early post-natal development of the rat ventral prostate (VP) (*Histochem Cell Biol* 129: 805, 2008). We then hypothesized that epithelial growth, branching, and canalization in the VP would require matrix remodeling, and hence MMP activity. Thus, we decided to evaluate the role of MMP-2 in the ventral prostate development either blocking MMP-2 using siRNA in whole organ culture and analysing morphological aspects of this gland in MMP-2 null mice. Inhibition of MMP-2 by siRNA compromised VP morphogenesis. MMP-2 silencing reduced organ size (on day 6) and epithelial area (on days 4 and 6), resulting in decreased number of epithelial tips. Histology and second harmonic generation (SHG) revealed that MMP-2 silencing affected VP architecture by interfering with lumen formation and cellular organization in both epithelium and stroma, paralleling intense accumulation of collagen fibers. The ventral prostate of adult MMP-2 null mice showed smaller relative weight, epithelial and smooth muscle cell volume, besides accumulating of reticular and collagen fibers. MMP-2 null neonate mice (day 6) showed lower cell proliferation rate and also reticulin fiber accumulation in their mesenchymal/stromal compartment. No difference in cell proliferation was noted between adult MMP-2 null and wild type (C57BL/6) mice. In conclusion, MMP-2 plays an important role in ventral prostatic growth, being directly involved with epithelial morphogenesis and acquisition of normal adult histology.

Keywords: *development, MMP-2, ventral prostate*

07.3- Type I and Type III Collagen and Elastic Fibers Appearing During Early Post-Natal Rat Prostate Development

Carolina Sarobo², Flávia Karina Delella¹, Elaine Manoela Porto Amorim¹, Larissa Mayumi Ribeiro², Livia Maria Lacorte², Justulin Jr Luis Antonio², Felisbino Sergio Luis²

1. UNICAMP, State University of Campinas, Cidade Universitária Zeferino Vaz, Monteiro Lobato, 255 - Campinas/SP/Brazil. 2. UNESP, Sao Paulo State University, Department of Morphology, Institute of Biosciences, Botucatu/SP/Brazil

The process of branching morphogenesis in rats and mice will give rise to three distinct bilaterally symmetrical prostatic lobes: the anterior prostate (AP), the dorsolateral prostate (DLP), and the ventral prostate (VP). Concurrent with the process of ductal branching morphogenesis, epithelial and mesenchymal/stromal differentiation occurs in the first 2–3 weeks after birth in rats and mice. Herein, we investigated the dynamic of extracellular matrix fibers deposition, such as type I and type III collagen fibers and elastic system fibers, in the rat prostate stroma during the first 4 weeks of the postnatal development. Wistar rats (n=16) were killed by overdose of pentobarbital (30mg/kg) after 7, 14, 21 and 28 postnatal days. The prostate complex together with the bladder and urethra were excised, fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr, washed, dehydrated, cleared, and embedded in Paraplast Plus embedding medium. Sections of 5 µm were stained by Weigert's Resorcin-Fuchsin and some sections were processed for immunohistochemistry for type I collagen (Sigma, C2456, Clone COL-1) and type III collagen (Sigma, C7805, Clone FH-7A). Type I collagen fibers appeared around prostatic epithelial cords and in the interstitium at the first week and a major amount were identify in the next weeks, with thicker fibers appearing organized around the ducts in the fourth week. Delicate type III collagen fibers were identified at the second week filling the stroma in a disorganized distribution and after 21 and 28 days of the postnatal development were organized restricted surrounding the ducts. Very thin elastin containing fibers were observed in the prostate stroma only around the ducts and in the interstitial stroma at the second week. At the third and fourth weeks these fibers became thicker and organized in a bigger amount around the ducts. In conclusion, this study showed that distinct extracellular matrix fibers deposition occurs simultaneously with the mesenchyme cytodifferentiation in fibroblasts and smooth muscle cells and at beginning of the glandular secretory function in the rat prostates.

Keywords: collagen fibers, elastic fibers, post-natal development, prostate

07.4- Fetal Programming Impairs Glandular Maturation and Decreases Matrix Metalloproteinases 2 and 9 Activity in Rat Ventral Prostate

Luis Antonio Justulin Jr, Jaqueline de Carvalho Rinaldi, Livia Maria Lacorte, Larissa Mayumi Ribeiro, Carolina Sarobo, Sérgio Alexandre Alcântara dos Santos, Patrícia Aline Boera, Sérgio Luis Felisbino

1. UNESP, Department of morphology, IB, Sao Paulo State University, District of Rubiao Jr, Botucatu, Sao Paulo

Fetal programming has been associated with impairment of fetal growth and development. Although the majority of studies of developmental programming have investigated the cardiovascular system, recently studies focusing the reproductive system demonstrated changes in some reproductive parameters such as: reduction in serum testosterone, in testicular weight and delay of testicular descent. Thus, the aim of this study was to investigate the effects of fetal programming induced by in utero protein restriction on rat ventral prostate morphophysiology. Male Wistar rats with underwent in utero fetal programming by protein restriction (normal diet=17% protein; protein in restricted diet=6%) was killed at age of 16 week and the ventral prostate was excised, weighted and processed for histology and gelatin-zymography analyzes. The programmed male offspring presented decrease in both absolute and relative ventral prostate weight compared to matched control. Histologically, the prostate showed ducts with reduced luminal area with few prostatic secretion accumulated into the ducts. Moreover, the interstitial stroma appears thicker compared to the control groups. The analyses of gelatin-zymography revealed decrease in both MMP-2 and MMP-9 activity in programmed rats, which can be correlated with stromal condensation. In conclusion, the fetal programming induced by protein restriction appears to delay the ventral prostate maturation and alters activity of enzymes involved with tissue remodeling.

Keywords: *fetal programming, MMPs, stroma, protein restriction, ventral prostate*

07.5- Intrauterine Estrogenization by Ethinylestradiol Disrupt the Development of Male and Female Adult Mongolian Gerbil Prostate

Ana Paula Silva Perez¹, Manoel Francisco Biancardi^{1,1}, Fernanda Cristina Alcântara dos Santos², Luiz Roberto Falleiros Junior², Rejane Maira Góes², Sebastião Roberto Taboga²

1. Unicamp, Universidade Estadual de Campinas, Distrito de Barão Geraldo, Campinas – SP. 2. IBILCE/UNESP, Instituto de Biociências, Letras e Ciências Exatas, Rua Cristóvão Colombo, 2265, Bairro JD Nazareth, São José do Rio Preto-SP

Estrogens play a physiological role during prostate development with regard to programming stromal cells and directing early morphogenic events. However, the estrogenic exposures are abnormally high during the critical developmental period, a process referred to as developmental estrogenization. Thus, the present study aimed to analyze the stromal aspects of male ventral prostate and female prostate of adult gerbil, both in normal condition and under the influence estrogenic agents. In addition, we verified the postnatal androgenic effects in these glands exposed to estrogenization during the development. Pregnant females received daily injections of ethinylestradiol (EE, 10µg/Kg) from the 17th until 19th day of pregnancy. The offspring, consisting of intrauterine estrogenized gerbils, received subcutaneous doses testosterone cipionate (1mg/Kg/ 48h), for 14 days, starting at the 105th postnatal day (EE/T group). When animals of EE and EE/T groups completed 120 days of age, they were sacrificed and male and female prostate fragments were processed for light microscopy and stained with Gömöri's reticulin. Immunohistochemistry for smooth muscle (SM) α -actin was also performed. Male and female prostates exhibited acini lined by a simple epithelium varying from cubic to tall prismatic profile and inserted into fibromuscular stroma.

In the EE and EE/T groups of male and female it was observed increase of collagen fibrils, mainly around lesions. This compared to control group of both prostates present accumulation of collagen fibrils in adjacent region to the epithelium. Furthermore, the data showed a disorder and increase of reticular fibers of treated groups of both prostates in relation to control group. The immunohistochemical study of SM α -actin revealed a decrease in muscle layer that surrounded the alveolus prostatic of male and female EE and EE/T groups compared to control. In other hand, the treated groups verified an increase of muscle layer in regions with lesions in epithelium of both prostates. In male and female EE/T group an absence of muscle layer was observed where epithelial buds are forming. The results showed alteration and apparent increase of stroma in regions with epithelial lesions of treated prostates, mainly with EE/T. Thus the findings of this study demonstrate the importance of estrogen action in the prostatic development of males and females and the additional action of androgens in the adult life.

Keywords: *Estradiol, Prostate, Estrogen, Intrauterine, Development*

07.6- Differential Matrix Metalloproteinases Activity (Mmps -2, -9 And -14) During Mice Endochondral Ossification

*Virginia Sant'Ana Cronemberger¹, Jose Mauro Granjeiro², Fabio Daumas Nunes¹,
Katiucia Batista Silva Paiva¹*

1. FOUSP, Faculdade de Odontologia da USP, Av. Lineu Prestes, 2227. 2. UFF, Universidade Federal Fluminense, Outeiro de Sao Joao Batista, sn

MMPs are zinc-dependent endopeptidases that, collectively, degrade all components of the ECM. They are able to remodel the ECM during normal processes such as embryogenesis and organogenesis, as well as in pathological processes such as tumor invasion. Our goal was to evaluate the temporal-spatial enzymatic activity of MMP-2, -9 and -14 in mice embryos and newborns during endochondral ossification. Femurs (n=5/period) were collected from foetuses (E13-E20) and 1 day postnatal (PN1), cryosectioned (8 μ m) and stored at -80°C. For in situ zymography, three FITC-conjugated substrates were used (DQ-gelatin, DQ-collagen type I and IV – 1/10 dilution, Molecular Probes) diluted in reactive buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 0.2 mM NaN₃, 0.5% low melting agarose, 0.03% Triton-X 100, 1% DAPI, pH 7.6), for 24-26 hours at 37°C. Negative controls were performed using general inhibitor (100 mM EDTA), MMP inhibitor (10 mM phenantroline) and synthetic MMP inhibitor (SB-3CT, 16 nM, 180 nM for MMPs -2 and -9, and 900 nM for MMP-14, respectively). Our results demonstrate general gelatinolytic activity more intense than general collagenolytic activity in all periods evaluated. During chondrocyte differentiation (E13), fluorescent areas were observed at chondrocytes and perichondrium. At cartilaginous anlagen (E14), enzymatic activity was restricted to bone core and proliferative chondrocytes. After vascular and cellular invasion of cartilaginous anlagen (E15) enzymatic activity was also detected at the center of cartilaginous anlagen in hypertrophic chondrocytes. From E16 to PN1, both gelatinolytic and collagenolytic activities were found in the growth plate. Using synthetic MMP inhibitor, the differential MMP activity was observed in all periods evaluated and in all substrates used. Taken together, our findings showed, for the first time, the

differential MMP activity, and that MMP-2, -9 and -14 play an important role during endochondral ossification, mainly at early stages and at the primary bone formation. Financial Support: FAPESP

Keywords: *MMP, in situ zymography, Endochondral Ossification*

08. ECM AND NEUROBIOLOGY

08.1- Astrocytes Treated by Lysophosphatidic Acid Induce Axonal Outgrowth by Modulating the Amount of Extracellular Matrix Proteins

Tania Cristina Leite de Sampaio e Spohr, Rômulo Sperduto Dezone, Stevens Kastrup Rehen, Flávia Carvalho Alcantara Gomes

1. UFRJ, Federal University of Rio de Janeiro, Av. Brigadeiro Trompowsky s/nº - CCS - bl F- sala F15

Introduction: Lysophosphatidic acid (LPA) is a potent lipid mediator that evokes a variety of biological responses in many cell types via its specific G protein-coupled receptors. LPA plays important roles in many biological processes such as brain development, oncogenesis, wound healing, and immune functions. We have previously demonstrated that astrocytes primed by LPA induce neuronal commitment of cerebral cortical progenitors *in vitro* (de Sampaio e Spohr et al., *J.Biol.Chem.*, 2008). Objective: In the present work, by using a neuron-astrocyte coculture model, we examined possible mechanisms behind the neurite outgrowth induced by LPA-treated astrocytes. Results: We showed that LPA, through astrocytes, increases neuronal differentiation, arborization and neurite outgrowth of developing cortical neurons. Treatment of astrocytes with LPA or with conditioned medium derived from LPA-treated astrocytes (LPA-CM) yielded similar results, suggesting the involvement of an astrocyte-derived soluble factor induced by LPA. Further, LPA-CM or LPA astrocyte treatment led to an increase in the extracellular matrix (ECM) proteins laminin and fibronectin. Such increase in ECM protein levels as well as astrocyte permissiveness to neurite outgrowth was reversed by inhibitors of the mitogen-activated protein (MAP) kinase (MAPK) or protein kinase A (PKA) signaling pathways. Conclusions: We provided evidence that LPA induces neuronal fate commitment and increases the complexity of neuronal arborization by modulating the content of ECM proteins in the surface of astrocytes. Our data reveal an important role of astrocytes as mediators of bioactive lipids action in brain development and implicate ECM and MAPK and PKA pathways in this process. Financial Support: FAPERJ, CNPq, CAPES

Keywords: *lysophosphatidic acid, neuron-glia interaction, extracellular matrix proteins, axonal outgrowth*

08.2- TGF-Beta 1 Promotes Cortical Synaptogenesis in Vitro

Juliana de Carvalho Almeida, Luan Pereira Diniz, Rogério Panizzutti, Flávia Carvalho Alcantara Gomes

1. UFRJ, Universidade Federal do Rio de Janeiro, 21949-590

Neuron-astrocyte interactions play critical roles in several events during central nervous system (CNS) development such as neuronal survival and differentiation, cell fate specification, modulation of formation and efficacy of synapses. One of the first identified synaptogenic glia-derived molecule was the extracellular matrix protein, thrombospondin. However, the mechanism underlying this event remains unknown. Recently, the transforming growth factor beta 1 (TGF-beta1), a cytokine that binds and is activated by thrombospondin, has been shown to promote synaptogenesis in the peripheral nervous system. Here, we investigated the role of TGF-beta1 as a mediator of thrombospondin effects in synapse formation in cerebral cortex neurons in vitro. Cerebral cortical neurons from 14 days embryonic mice were treated with TGF-beta1 (10ng/ml) and maintained for 12 days in culture. Synapse formation was evaluated by immunocytochemistry labeling against the pre-synaptic protein, synaptophysin and the post-synaptic protein, PSD-95. TGF-beta 1 dramatically increased the levels of either synaptophysin (100% increase) and PSD-95 (122%). These data were supported by western blot assays that revealed 60-70% increment in the levels of both proteins in TGF-beta1-treated neurons. Together, our results show a new role for TGF-beta 1 as a synaptogenic molecule in CNS and suggest a mechanism underlying thrombospondin induced-synaptogenesis. This work was supported by CAPES, CNPq and FAPERJ.

Keywords: *TGF-beta1, D-serine, synaptogenesis*

08.3- Role of MAPK and PI-3K Pathways Activated by TGF-Beta 1 on Radial Glia-Astrocyte Transformation in Vitro

Daniel Francis, Joice Stipursky, Flávia Carvalho Alcantara Gomes

1. UFRJ, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, bloco F, F15

Objective: The major progenitor of the developing cerebral cortex is the radial glial (RG) cell, which originates neurons and glial cells. We previously described that the transforming growth factor beta 1 (TGF-beta1) promotes RG-astrocyte transformation in vitro. TGF-beta1 actions are mainly triggered by its receptors, TGFRI and TGFRII, followed by phosphorylation and nuclear translocation of the transcription factors, SMADs 2 and 3. Alternatively, TGF-beta1 might activate non-canonic pathways such as MAPK (mitogen activated kinase protein) and PI3K (phostatidilinositol-3-kinase), although the contribution of these signaling pathways to astrocyte differentiation is less known. Here, we analyzed the role of MAPK and PI3K on astrocyte differentiation induced by TGF-beta1 in vitro. **Methods and Results:** Primary RG cell cultures derived from cerebral cortex of 14 days (E14) embryos were maintained for 24h with DMEM-F12 medium supplemented with TGF-beta1 (10ng/mL)

and/or the inhibitors of TGFRI (SB431542, 10uM), PI3K (LY294002, 5uM) and MAPK (PD98059, 50uM). Cell differentiation was analyzed by immunocytochemistry for the markers: BLBP, (RG cell), GFAP (astrocyte), Nestin (neural progenitors) and betaTubulinIII (neuron). The immunocytochemistry analysis revealed a 43% increase in the number of GFAP+ cell in response to TGF-beta1 treatment. This event was reversed by the MAPK inhibitor, PD98059. These data were supported by western blotting of RG cell cultures: whereas treatment of RG cultures with TGF-beta1 increased the levels of phosphorylated MAPK, simultaneously addition of TGF-beta1 and PD98059 inhibited Erk phosphorylation. The number of Nestin+/BLBP+ was decreased by 36%, and this event was reversed by the PI3K inhibitor, LY294002. TGF-beta1 treatment decreased the number of betaTubulinIII by 18%, and this effect was reversed by both inhibitors. Conclusions: These data suggest that TGF-beta1 might act through distinct pathways to determine cell fate: through MAPK, on astrocyte generation, through PI3K, on the maintenance of RG progenitor phenotype, and through both pathways, on neuronal generation. Financial support: FAPERJ, CNPq, CAPES.

Keywords: *Astrocyte, Radial glia, TGF-beta1*

08.4- TGF-Beta 1 Promotes Differentiation of Radial Glia Into Astrocyte and Disrupts Basal Membrane in Cerebral Cortex in Vivo

Joice Stipursky³, Daniel Francis³, Eva Anton², Flávia Carvalho Alcantara Gomes³

2. UNC, University of North Carolina at Chapel Hill, Neuroscience Building Mason Farm Rd 7109B, Chapel Hill, North Carolina, EUA. 3. UFRJ, Universidade Federal do Rio de Janeiro, Av. Carlos Chaga Filho, 373, bloco F, sala F15

The major cerebral cortex stem cell population present in the developing brain is the radial glial cell (RG), which originates neurons and glial cells. RG cells present a bipolar morphology, which is maintained by anchoring its pial process in the basal membrane structure that covers the brain surface. The mechanisms that modulate RG progenitor morphology maintenance or its differentiation into specific cell types are not completely known. Recently, we showed that the transforming growth factor beta 1 (TGF- β 1), which is a regulator of extracellular matrix (ECM) protein synthesis and deposition, promotes RG into astrocyte differentiation in vitro. Here, we analyzed the potential of TGF- β 1 in RG-astrocyte transformation in vivo and the involvement of basal membrane in this event. Pregnant Swiss female mice (14 days) were anesthetized and the embryos were subjected to intraventricular injections of TGF- β 1 (100ng) followed by an intraperitoneal injection of BrdU solution (10mg/mL) 1h later. After 48h, the female was sacrificed, the E16 embryos removed and the brains collected and fixed in 4% paraformaldehyde. Brains were vibratome-sectioned and processed for immunohistochemistry with antibodies against RG markers (BLBP and Nestin), astrocytes (GFAP) and the pial surface basal membrane of the brain (Laminin). The TGF- β 1-injected cortex presented huge changes in RG cell morphology, characterized by the loss of the typical process bearing morphology of RG in 60% of BLBP+ cells and a 90% increase in the number of GFAP+ cells. Number of GFAP/BrdU cells was not affected by TGF- β 1. This event was followed by a robust disruption of basal membrane, characterized by ectopic Laminin+ cells throughout the cerebral cortex. Conclusions: Our data show that RG

differentiation into astrocytes induced by TGF- β 1 might be associated to reorganization of the ECM protein, Laminin, suggesting a novel role for this molecule in cerebral cortex development. Financial support: FAPERJ, CNPq, CAPES.

Keywords: *astrocytes, cerebral cortex, radial glia, neural stem cell, TGF-beta 1*

08.5- Astrocytes as Mediators of Thyroid Hormones Actions in Neuronal Differentiation and Neurite Outgrowth: Role of Chondroitin Sulfate

Rômulo Sperduto Dezonne¹, Joice Stipursky¹, Jader Nones¹, Mauro Sérgio Gonçalves Pavão², Flávia Carvalho Alcantara Gomes¹

1. UFRJ, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, Bloco F, Sala F15. 2. UFRJ, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, HUCCF, 4o andar, sala 4A08

Introduction: Thyroid hormones, thyroxin (T4) and 3, 5, 3'-triiodothyronine (T3), are essential for a wide range of processes of mammalian brain development, such as neuronal migration, neural cells differentiation and myelination. Some of THs actions are mediated by astrocytes, though the mechanism of action underlying this event is still controversial. Here we investigated the interplay between THs action and glycosaminoglycans (GAGs) in neuron-astrocyte interactions in vitro. **Experimental Procedures:** Astrocytes primary cultures were prepared from cerebral cortex derived from newborns Swiss mice, in DMEM/F12 supplemented with 10% of fetal bovine serum. After reaching confluence, the cultures were incubated an additional day in medium without serum followed by three days-treatment with 50nM of T3 or T4. Progenitor cells derived from 14 days embryonic (E14) cerebral cortex were cultured either onto treated astrocyte monolayers or in the presence of their conditioned medium (CM). To analyze a possible influence of GAGs, cultures were enzymatically digested with chondroitinase AC 2h prior addition of progenitor cells. After 24h of coculture, neuronal differentiation was analyzed by immunocytochemistry assays against the neuronal marker, α -tubulin III, and morphometrical analysis. **Results:** T3- and T4-treated astrocytes increased neuronal population by 36% and 50%, respectively. In addition, THs astrocyte treatment enhanced neurite length by 73% and increased neuronal arborization as revealed by doubling the number of neurons with two and three neurites. Surprisingly, treatment of astrocyte monolayers with chondroitinase AC completely inhibited these effects. Similarly, culture of E14 progenitor cells in THs treated-astrocyte CM yielded a 48% increase in neurite length, in both conditions; although only T4-CM enhanced neuronal population. **Conclusions:** Our results show that cerebral cortex astrocytes treated by THs increase neuronal differentiation and maturation through modulation of extracellular cellular components. Our data clearly suggest the involvement of chondroitin sulfate glycosaminoglycans as key mediators of THs actions during neuron-astrocyte interactions in the cerebral cortex. **Financial Support:** FAPERJ, CNPq and CAPES.

Keywords: *astrocytes, glycosaminoglycans, neuronal differentiation, neuron-glial interactions, thyroid hormones*

08.6- Flavonoids and Astrocytes Crosstalking: Implications for Brain Development and Pathology

Jader Nones, Flávia Carvalho Alcantara Gomes

1. UFRJ, Federal University of Rio de Janeiro, UFRJ, Institute of Biomedical Science, Laboratory of Cellular Neurobiology, F14

Introduction: Flavonoids are naturally occurring polyphenolic compounds present in a variety of fruits, vegetables, cereals, tea and wine. Evidences have suggested that these phytochemicals might have an impact in brain pathology and aging, however, either their mechanisms of action are not completely known. We previously demonstrated that astrocytes treated by the flavonoid casticin increased cerebral cortex neurons by modulating the pool of neuronal progenitors (Spohr et al., J.Neurosci.Res., 2009). **Objective:** In the present work, we performed a screening of flavonoid actions by analyzing the effects of these substances (hesperidin, quercetin and rutin) on murine cerebral cortex astrocytes and neural progenitors. **Methodology:** Neural progenitor cultures from 14 days embryonic mice (E14) were treated with flavonoids (10 μ M) and maintained for 2 days in culture. The indirect effect of flavonoids, via astrocytes, was evaluated by culturing E14 progenitors for 48 hours in the presence of conditioned medium (CM) from flavonoid-treated astrocyte. In both cases, direct and indirect assays, neuronal, astrocytic and oligodendrocytic differentiation was evaluated by immunocytochemistry against specific cellular markers. Cell proliferation and cell death were analyzed by Ki67 and caspase 3-immunolabelling, respectively. **Results:** Treatment of neural progenitors with hesperidin enhanced neuronal population as revealed by 75% increase in the number of beta-tubulin III cells. This effect was mainly due to modulation of neuronal progenitor survival by decreasing cell death. Pools of astrocyte and oligodendrocyte progenitors were not affected by hesperidin. Culture of neural progenitors with CM from hesperidin treated-astrocyte yielded similar results, suggesting the involvement of an astrocyte-derived soluble factor. Quercetin and rutin had no effect on neuronal population. Cellular differentiation, proliferation and neuronal complexity were not affected by any of the flavonoids used. None of the flavonoids affected astrocyte morphology and proliferation. **Conclusions:** Together, our data suggest that hesperidin influences neuronal population by two mechanisms: 1) directly, by decreasing neuronal death; 2) indirectly, via astrocytes, by modulating the pool of neuronal progenitors. Grant Support: FAPERJ, CNPq, CAPES.

Keywords: *Astrocytes, Flavonoids, Neural progenitors*

08.7- Retinal Vascular Abnormalities in PRPC Knockout Mice

Mônica Moreau da Cunha Lima^{1,2}, Cristiane Lima Bicalho de Barros¹, Tatiana Coelho-Sampaio¹, Rafael Linden¹, Elisabete Freire¹

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Brigadeiro Trompowsky s/número - Ilha do Fundão CCS Bloco B. 2. UNIJORGE, Centro Universitário Jorge Amado, Av. Paralela s/número Salvador

Prion scrapie (PrPsc), the altered form of cellular prion protein (PrPc), is the infectious particle of a class of diseases named spongiform encephalopathies. However, the physiological function of PrPc has not been established so far. In this work, we prepared retina whole mounts of newborn PrPc knockout and wild-type mice and labeled them with DAPI for nuclei labeling, anti-laminin, anti-type IV collagen and anti-PECAM-1 antibodies in order to investigate possible structural alterations associated to the lack of PrPc expression in the eye. Laminin polymers observed in wild-type mice displayed polygonal patterns, comparable to those previously described in embryonic mice astrocyte in culture [Freire et al. (2004) J. Cell Sci. 117: 4067-4076]. This polygonal pattern was not observed in knock-out mice, which is compatible with PrPc as being a laminin receptor. In addition, several morphological changes were observed in knockout mice, namely: 1) decrease in vessel diameter, 2) reduction in capillary branching, 3) loss of pericyte adherence and 4) an increase in the incidence of retinal detachment. We are currently examining ocular fundus in knockout and wild-type animals in order to analyze the vascular network in vivo. Our findings suggest that molecular, cellular, histological and functional particularities in mice retina may be correlated to PrPc function in vasculogenesis and vessel maturation in mammals. Supported by CAPES, CNPq and FAPERJ

Keywords: *PrPc, retinal vasculogenesis, knockout, visual function, laminin*

08.8- Interaction between a Specific Ganglioside and Integrins During Neuronal Migration in Rat Developing Cerebellum

Virgínia Chaves de Lima Werneck de Castro, Gabriele Machado dos Santos, Marcelo Felipe Santiago, Rosalia Mendez Otero

1. IBCCF, Instituto de Biofísica Carlos Chagas Filho, Av. Carlos Chagas Filho, n 373
edifício CCS Bloco G

During development of nervous system there are some important steps occurring such as cell proliferation and migration. In concerning to neuronal migration during cerebellum development, a number of molecules have been studied to understand the possible signaling pathways involved in this event. One of these molecules is the ganglioside 9-O-acetyl GD3 which immunoblockade by monoclonal antibody Jones leads to a significant decrease in neuronal migration either in cerebral cortex and cerebellum. One of our hypotheses is the interaction of this ganglioside to integrins. Based on this idea, the ganglioside may be a possible candidate as a modulator of integrins related signaling pathways. To address these questions we performed rat cerebellar microexplants cultures and antibody perturbation experiments to assess the influence of integrins on neuronal migration. We also performed immunoprecipitation assays to ensure the link between beta 1 integrin and 9-O-ac GD3 in our model as well as confocal microscopy analysis of immunocytochemical experiments. Sucrosis gradient was performed to identify the possible location of 9-O-acetyl GD3 within membrane microdomains. We observed that in our model, the blocking peptide against alpha 6 integrin is capable to arrest the migration. These data was analyzed by T test comparing control cultures to treated ones. Alpha 6 and beta 1 integrins subunits co-localize with 9-O-acetyl GD3 in culture explants and the ganglioside immunoprecipitates the beta 1 integrin

subunit in our cultures. In sucrosis fractionating experiments we find 9-O-acetyl GD3 in low density fractions indicating its possible participation in specific membrane microdomains. We can partially conclude that 9-O-acetyl GD3 ganglioside is linked to the beta 1 integrin in our model suggesting a modulation of its activity, maybe altering integrin affinity for laminin. Once in cerebellum the beta 1 subunit can be associated to alpha 6 subunit, we showed that blocking alpha 6 subunit with blocking peptides have led to inhibition of migration. These molecules can be associated to each other within specific membrane microdomains facilitating the triggering of signaling pathways. Further experiments will be necessary to evaluate the involvement of specific molecules such as FAK and src-c, key molecules in integrin signaling pathway.

Keywords: *gangliosídeos, migração neuronal, integrinas*

08.9- Effects of Epidermal Growth Factor on Cerebellar Glia

Cristiane M. Eller, Rodrigo Martinez, Nathan Viana, Flávia Gomes

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Brigadeiro Trompowsky s/n,
Bloco F, sala F-15, CCS, Ilha do fundão

Two glial cell population play pivotal role during cerebellar development: Bergmann glia (BG) cells, responsible for guiding neuronal migration and astrocytes, the main source of trophic factors for granular neurons. Here, we analyzed the effect of epidermal growth factor (EGF) in cerebellar glial development and neuronal migration in vitro and the signalling cascades involved in these events. Cerebellar astrocytes from newborn rats were cultured for 10 days in vitro in DMEM/F12 medium, supplemented with 10% fetal bovine serum. After reaching confluence, cells were washed and maintained for additional 2 days in the presence of 10 ng/ml of EGF. For BG studies, cultures from 7 days old rats cerebella were maintained for 3 days in vitro in the previous medium, followed by 2 days in the presence of 10 ng/ml of EGF. Morphometric analyses of BG were performed by imunocytochemistry for the glial marker glial fibrillary acidic protein (GFAP). Levels of extracellular matrix proteins (ECM) of astrocytes were evaluated by immunocytochemistry for laminin and fibronectin. Signaling pathways were analyzed by using different signaling cascade inhibitors: PD98059 (MAPK; mitogen-activated protein kinase), KT5720 (PKA; protein kinase A), Staurosporin (PKC; protein kinase C) and LY294002 (PI3K; phosphatidylinositol-3-kinase). Treatment of cerebellar astrocytes with EGF strongly induced synthesis and secretion of ECM proteins, fibronectin and laminin. Similarly, EGF greatly induced BG elongation (increase by 50%), an essential condition for neuronal migration. Both effects, in BG and astrocytes, were impaired by the MAPK inhibitor. To address neuronal migration, explants from 7 days old rats cerebella were kept on laminin in the presence of EGF (10 ng/ml), and cell migration was analyzed after 48hr. After this period, EGF increased by 30% the distance of cell migration. Time lapse microscopy assays suggested that the effect of EGF in neuronal migration is delayed since EGF did not alter either neuronal velocity or mobility after short-term (2hr) treatment. Neuronal migration induced by EGF was inhibited by the MAPK inhibitor, PD98059. Our results suggest that EGF through MAPK pathway is an important modulator of glial biology

in the cerebellum: 1) EGF induces synthesis and secretion of ECM proteins by astrocytes; 2) EGF induces neuronal migration and BG elongation. Grant Support: FAPERJ, CAPES and CNPq.

Keywords: *Bergmann glia, Cerebellum development, Epidermal Growth Factor*

08.10- Prion Protein Transduces Signals after Binding to Laminin Gama1 Chain Via Metabotropic Glutamate Receptors

Flavio H. Beraldo^{1,3}, Camila Arantes^{1,2}, Tiago Goss dos Santos¹, Cleiton Machado¹, Ana Magalhães³, Margareth Magdesian⁴, Stephen S.G. Ferguson³, Rafael Linden⁴, Marco A. M. Prado³, Vilma R. Martins¹

1. LICR/SAO PAULO, Ludwig Institute for Cancer Research, São Paulo Branch, Rua João Julião, 245 1º andar. 2. IQ/USP, Instituto de Química, Universidade de São Paulo, Universidade de São Paulo, São Paulo. 3. ROBARTS, Robarts Research Institute, and Department of Physiology, University Western Ontario, London, ON, Canada. 4. IBCCF, Instituto de Biofísica Carlos Chagas Filho, UFRJ, Universidade Federal do Rio de Janeiro, Rio de Janeiro

The prion protein (PrP^C) is a cell surface glycoprotein that is highly expressed in the nervous system whose conformationally modified isoform is responsible for prion diseases. Multiple and diverse functions of PrP^C have been reported. PrP^C interacts with a peptide at the carboxi-terminal of the laminin γ 1 chain (RNIAEIIKDI) promoting neuronal survival and neuritogenesis. Using an intracellular Ca²⁺ probe, Fluo- 3 AM, we verified that the laminin γ 1 peptide, in particular its KDI domain, increased intracellular Ca²⁺ in wild-type neurons whereas no effect was observed in PrP^C-null neurons. Neuritogenesis as well as Ca²⁺ signaling were abrogated by U73122, a specific inhibitor of the phospholipase C (PLC) and by 2APB, a specific inhibitor of inositol 3-phosphate receptor at the endoplasmatic reticulum. Remarkably, inhibition of a group I metabotropic glutamate receptor (mGluR1) with LY367385 decreased the effect of PrP^C-laminin γ -1 peptide interaction upon mobilization of intracellular Ca²⁺, and blocked the activation of PKC. The expression of mGluR1 or mGluR5 receptors in HER293 cells reconstituted the signaling pathways mediated by PrP^C- laminin γ 1 peptide interaction. These data established that group I mGluRs are key molecules promoting PrP^C-laminin neurotrophic properties. Moreover, our data suggest that Ca²⁺ is the first intracellular signal activated by PrP^C- laminin γ -1 peptide interaction which is followed by consecutive PKC, AKT and ERK activation which finally promotes neuritogenesis.

Keywords: *calcium, glutamate receptors, laminin, prion protein*

08.11- Impairment in Ca²⁺ Signaling Mediated by Laminin in Neurons Expressing Mutant Prion Proteins Associated with Genetic Prion Diseases

Cleiton Fagundes Machado^{1,2}, Flavio Henrique Beraldo¹, Dominique Bourgeon¹,

Vilma Regina Martins¹

1. LICR, Ludwig Institute for Cancer Research, Rua João Julião, 245 1A Bela Vista 01323-903, São Paulo SP Brasil. 2. AC Camargo, Centro de Tratamento e Pesquisa do Hospital A.C. Camargo, Rua Professor Antonio Prudente, 109 Liberdade, 01509-010 São Paulo SP Brasil

Prion protein (PrPC) is a GPI anchored protein mostly expressed at the surface of neuronal and glial cells. Mutations in the PrPC gene are involved in neurodegenerative disorders called Transmissible Spongiform Encephalopathies (TSEs) or genetic prion diseases. It is still under debate if PrPC loss-of-function is, at least for a part, responsible of the pathogenic process of these diseases. Some PrPC functions have been associated with its ability to bind specific cellular proteins. We previously demonstrated that the region between aminoacids 173 to 182 of PrPC binds to a peptide (RNIAEIIKDI) at the laminin gamma1 chain promoting neuritogenesis and memory formation. A 3 fold increase in the intracellular calcium concentrations was observed in primary neuronal cultures derived from wild-type mice upon treatment with laminin gamma1 peptide while any effect was observed in PrPC-null neurons. The expression of PrPC in an immortalized cell line derived from PrPC-null neurons (CF10) rescued calcium signaling responses mediated by PrPC-Laminin gamma1 peptide interaction. Three PrPC mutations associated with genetic Creutzfeldt-Jakob disease and Fatal Familial Insomnia map within the laminin gamma1 binding site. In order to evaluate PrPC-loss-of-function, cDNAs coding mouse PrPC molecules with the equivalent human mutations were inserted into pCDNA3 and expressed in CF10 cells. The expression of PrPC mutants 177N, 179I and 182A as well as the wild-type protein was confirmed by flow cytometry. Cells expressing the wild-type or mutant proteins were labeled with the intracellular calcium probe Fluo 3 AM, treated with laminin gamma1 peptide in the presence of extracellular calcium. Data acquisition for intracellular calcium was performed with a confocal microscope Bio-Rad Radiance 2100/Nikon. Cells expressing the mutations 177N or 182A exhibited a similar increase in intracellular calcium when compared to the wild-type PrPC. Conversely, the PrPC mutation 179I, presented 30.5 ± 1.1 % lower intracellular calcium levels upon laminin gamma1 peptide interaction compared to wild-type PrPC. These data suggest that PrPC mutation at codon 179 causes a partial impairment in calcium signaling mediated by its association with laminin. The impact of this impairment on neuronal plasticity and its possible participation in the disease pathogenesis is under investigation.

Keywords: *Prion Protein, Laminin, Calcium*

08.12- Flavonoids from Croton Betulaster Induces Glial and Neuronal Differentiation of Human Glioblastoma Multiform Cells and Changes the Pattern of Fibronectin Expression.

Cleide dos Santos Souza, Paulo Lucas Cerqueira Coelho, Mona das Neves Oliveira, Milton Albuquerque Franco Souza, Lilian Lessa Andrade, Sandra Regina Villas-Boas Freitas, Pedro Rocha Barbosa, Catia Suse de Oliveira Ribeiro, Marcienne Tardy, Silvia Lima Costa

1. UFBA (LabNq), Universidade Federal da Bahia, Lab. Neuroquímica e Biologia Celular ICS-UFBA Av.Reitor M.Calmon,Salvador,Brazil

Glioblastomas are rapidly proliferating malignant tumours, particularly resistant to classic antitumor treatment. Flavonoids derived from plants are active compounds responsible for various biological effects including antitumoral activity. Our previous study demonstrated that flavonoids isolated from the Brazilian plant *Croton betulaster* (acacetin, casticin, apigenin and pendulitin) can inhibit the growth of human glioblastoma multiform cell line GL-15, and down regulated TGFbeta secretion. In this study we investigated the effect of apigenin and pendulitin, the most active flavonoids from *C. betulaster*, on morphogenesis, and expression of ECM component fibronectin in human glioblastoma cells. Synchronized GL-15 and U251 cell lines, derived from human multiform glioblastomas, were cultured in supplemented DMEM on a ECM support of laminin and treated with flavonoids (50µM) for 48 h, or with the vehicle DMSO (0.1%). Immunocytochemistry assays show that glioblastoma cells in control conditions present a bipolar phenotype and express constitutively nestin, the marker of precursors cells, and low levels of GFAP (astrocyte marker) and betaIII-tubulin (neuronal marker). We observed that in both glioblastoma lineages nestin levels were down regulated after flavonoids exposure. On the other hand a subpopulation of U-251 and Gl-15 cells presented a multipolar phenotype with over expression of GFAP, charactering astroglial differentiation, and another subpopulation presented a filamentar phenotype with long cellular process expressing betaIII-tubulin, charactering neuronal differentiation. Moreover, beside neuroglial differentiation, we observed that flavonoids induced over expression of fibronectin in glioblastoma Gl-15 and U-251 cells. These results indicates that flavonoids extracted from *C. betulaster* present antitumoral and morphogenic activity to glioblastoma cells, and suggests these molecules as promising supplementary drugs for glioblastoma treatment.

Keywords: *Glioblastoma, Flavonoids, Differentiantion, Fibronectin, ECM*

08.13- Effects of A Spinal Cord Lesion on the Proliferation of Subventricular Zone Precursors of Adult Rats

Ana Cristina Machado Leão, Raquel Maria Pereira Campos, Ana Maria Blanco Martinez, Claudia Domingues Vargas, Cecilia Hedin Pereira

1. UFRJ, Uiversidade Federal do Rio de Janeiro, Av Brigadeiro Trompowski s/n CCS - Bl.F - F1-025

Spinal cord injuries (SCI) promotes apoptosis on sensorimotor cortex of adult rats. Apoptosis induced by specific lesion of neurons on cortical layer V stimulates the formation of new neurons by the subventricular zone of the lateral ventricles (SVZ). Also, a SCI promotes extensive sensorimotor cortex reorganization. On the present study, we investigated if a model of SCI that induces cortical apoptosis and plastic reorganization also modulates SVZ proliferation. Adult Wistar rats were subjected to laminectomy at T9 and compression with a vascular clip (30g 2 min). We analyzed two groups: injured (n=4) and sham (n=3) and 2 survival times: 1 and 5d. This model of compression causes a moderate injury and the

animals showed hindlimb paralysis. As a control of the injury, the animals were analyzed for their motor behavior (BBB test). 2h after the surgery, all animals received an injection of proliferation marker BrdU. The animals sacrificed 1d after injury received 3 BrdU injections. For the 5 days survival group, the BrdU injections were daily. All animals were sacrificed 2h after the last injection. The brains and spinal cords were dissected and prepared for immunohistochemistry or stained for HE or Kluver-Barrera. The brains were analyzed as anterior (1.20-1.00; 0.70-0.48; 0.20mm) or posterior SVZ (-0.26; -0.30-0.40; -0.80-0.92mm) relative to Bregma. For each ventricle, all BrdU+ cells on the lateral wall were counted under fluorescence microscope. We found that 5d after injury, experimental animals had more BrdU+ cells in both SVZ regions when compared to control animals (unp t test; antSVZ: control 137.5 ± 4.66 ; exp 160.9 ± 4.94 , P

Keywords: *neurogenesis, spinal cord lesion, cortical plasticity*

08.14- Changes in Heparan Sulfate Proteoglycan Expression Pattern During Early Cerebellar Development

Ana Paula Bérqamo Araujo, Maria Emília O. Brenha Ribeiro, Ritchelli Ricci, Ricardo José S. Torquato, Leny Toma, Marimélia Pocionatto

1. UNIFESP, Depto. Bioquímica Universidade Federal de São Paulo, Rua Três de Maio, 100 Vila Clementino São Paulo-SP

The correct development of the CNS relies on accurate processes controlled by intrinsic programs as well as on extracellular cues. Glial cells play important roles in the development of the CNS although it is not completely understood how those cells influence neuronal precursors during brain development. Heparan sulfate proteoglycans are components of cell surface and extracellular matrix that participate in a wide range of events, including axonal guidance, synapse formation, and modulation of growth factor and morphogen signaling. We were interested in knowing if there are changes in the expression of the two major brain heparan sulfate proteoglycans, glypican and syndecan, during early postnatal development and if their expression is modulated by glial cells. Initially, we investigated the expression of four members of the syndecan family and six members of the glypican family, as well as heparan sulfate sulfotransferases, and extracellular sulfatases by cerebellar neuronal precursors using quantitative real time RT-PCR. Our data shows that, in isolated granule cell precursors, sindecans 2, 3 and 4 are upregulated overtime, and that glypicans 2, 3, 5 and 6 are downregulated from postnatal day 3 to 9. Also, the expression of the heparan sulfate sulfotransferases changed during early postnatal development, being up- or downregulated depending on the sulfotransferase analyzed. Both extracellular sulfatases evaluated are downregulated in the early postnatal period. In order to look at the influence of glial cells on neuronal precursors, we compared the expression of sindecans and glypicans by neuronal precursors treated with glial conditioned media. Our data show that glial cells release soluble factors that stimulate axonal outgrowth by cerebellar neuronal precursors in an age-dependent manner. Three-day old neuronal precursors treated with glial conditioned media upregulate the expression of syndecans and downregulate the expression of glypicans. The data presented here indicate that heparan sulfate proteoglycan expression is controlled during CNS

development and can be modulated by interactions between neuronal precursors and glial cells. Support: FAPESP, CNPq.

Keywords: *Cerebellum, Development, Glial Cell, Glypican, Syndecan*

08.15- Physical Activity Triggers Neuroplasticity Events in Mouse Cerebellum

Ana Paula Bergamo Araujo¹, Michele Schultz Ramos Andrade^{2,1}, Fabiana Sant'anna Evangelista², José Eduardo Krieger², Marimelia Porcionatto¹

1. UNIFESP, Universidade Federal de São Paulo, Rua 3 de Maio, 100. 2. USP, Universidade de São Paulo, Rua Arlindo Bettio, 1000

Purpose: Physical activity stimulates neuroplasticity in CNS. The cerebellum is involved with motor learning and control presenting a high neuroplasticity. In order to better understand the molecular and cellular mechanisms involved in the process, gene expression was evaluated using real time RT-PCR and protein expression was analyzed by immunofluorescence. The genes investigated in this study were the heparan sulfate proteoglycans syndecans 1-4, the axonal growth associated protein 43 (GAP43) and CD133, a marker for neural stem cell. **Methods:** Adult male C57/BL6 mice were randomly assigned to sedentary and trained groups. Physical training consisted of two 1.5-h daily sessions of swimming training, 5 days/wk, for 4 wk. Sedentary mice were placed in the swimming apparatus for 5 min twice/wk during the experimental protocol. Two animals from each group received BrdU injections at training days 3, 7, 14, 21 and 28. After the training period, animals were anesthetized and euthanized followed by removal of cerebella. Animals that received BrdU injections were perfused transcardially and the sectioned cerebella were submitted to immunofluorescence labeling using anti-BrdU and anti-CD133 antibodies. **Results:** No changes were found for GAP43 and CD133 gene expression of cerebella after training. Physical activity regime did not alter the expression of syndecan 1 and 4. However, the expression of syndecan2 was three times higher in trained group compared to sedentary (p

Keywords: *neuroplasticity, physical activity, stem cell, syndecans*

09. ECM AND CANCER

09.1- The Mechanism of Lymphocytes Heparanase Activation

Thérèse Rachell Theodoro¹, Helena Bonciani Nader², Maria Aparecida da Silva Pinhal^{1,2}

1. FMABC, Faculdade de Medicina ABC, Av. Lauro Gomes, 2000 SA. 2. UNIFESP, Universidade Federal do Estado de São Paulo, R. Três de Maio, 100 SP

Syndecan family corresponds to cell surface heparan sulfate proteoglycans and is related with cell adhesion, migration and proliferation. Heparanase-1 (HPA1) is an endo-beta-

glucuronidase encoded by 4q21,3 gene that specific degrades heparan sulfate (HS) chains from proteoglycans. HPA1 increases syndecan-1 shedding promoting angiogenesis, tumor growth and metastasis (Sanderson et al., 2007). The heparanase-2 (HPA2) isoform presents no enzymatic activity and the gene is located in the 10q23,24 chromosome. Previous studies have shown that serum or plasma from breast cancer patients enhances both heparanase isoforms expression (HPA1 and HPA2) in the healthy women lymphocytes (Theodoro et al., 2007). The aim of this study is to elucidate the mechanism of lymphocyte activation by tumor cells. In vitro assays demonstrated that co-culture medium obtained from a human breast cancer cell line (MCF-7) and healthy woman lymphocytes were also able to stimulate both isoforms of heparanases (HPA1 and HPA2), while MCF-7 conditioned medium had no effect. Using specific monoclonal antibody target to heparan sulfate proteoglycan F69-3G10 (Seikagaku Corporation, JAPAN) and digesting conditioned co-culture medium with heparitinases I and II from *Flavobacterium heparinum* it was elucidated that HS should be involved in the stimulatory effect upon lymphocytes. This data was confirmed using a monoclonal antibody against syndecan-1, MCA681-CD138 (Serotec Accurate Scientific, USA). Labeling lymphocytes with [35S]-sulfate it was confirmed that activated lymphocytes synthesized chondroitin sulfate and secrete dermatan sulfate and heparan sulfate. The results confirmed that lymphocytes heparanase isoforms expression may be stimulated by the presence of the tumor and be used as breast cancer marker. The mechanism of lymphocytes activation involves heparan sulfate signaling that is secreted by the tumor cells.

Keywords: *heparan sulfates, heparanase, heparitinases, lymphocytes, tumor*

09.2- Heparanase Expression and Sulfated Glycosaminoglycans Profile in Kidney Cell Carcinoma

*Sabrina Nascimento¹, Lucas Batista¹, Helena Bonciani Nader²,
Maria Aparecida da Silva Pinhal^{1,2}*

1. FMABC, Faculdade de Medicina do ABC, Av. Lauro Gomes, 2000 SA. 2. UNIFESP, Universidade Federal do Estado de São Paulo, R. Três de Maio, 100 SP

The incidence of kidney cell carcinoma has been rising around 4% each year. In the United States, the incidence reached 54390 new cases with 13010 deaths in 2008 (Jemal et al. 2008). Molecular biology and extracellular matrix (ECM) remodelling has an important role in the understanding of the structural alterations of kidney tumor cells, as well as in the precocious diagnosis. Increased expression of heparanase (HPA) is involved with tumor development and metastasis (Hullet et al. 1999; Vlodavsky et al. 2000; Staquicini et al., 1990). HPA is an endo-beta-glucuronidase that cleavages intradisaccharide heparan sulfate chains from proteoglycans and has been involved with tumor development and metastasis (Cohen et al. 1994). The main objective of this study was to determine HPA expression and the sulfated glycosaminoglycans (GAG) profile in the kidney cell carcinoma, compared to the non tumoral and transition tissues obtained from surgery. Real time PCR was performed to quantified both HPA isoforms, HPA1 and HPA2, using two housekeeping gene, the ribossomal protein RPL13a and GAPDH. Immunohistochemistry (IHC) was quantified by digital software ImageLab® using four specific antibodies to HPA1 and HPA2 isoforms

(Santa Cruz). GAG was identified and quantified by agarose gel electrophoresis and densitometry. HPA1 was significantly higher expressed in kidney cell carcinoma tissues compared to the non tumoral tissues. HPA1 and HPA2 isoforms presented the same IHC labeling intensity. However, the active isoform of HPA1 (HPA1 50 kDa) has shown higher index of positive cell number indicating that possibly this isoform could be related to carcinogenesis. GAG quantification revealed a decreased in heparan sulfate (3.6 ± 3.5), (17.6 ± 20.5) and dermatan sulfate (16.9 ± 21.7), (44.2 ± 28.5), respectively, for cell kidney carcinoma and non tumoral tissues, while chondroitin sulfate was increased in cell kidney carcinomas (50.6 ± 37.2), compared to the non tumoral tissues (10.0 ± 9.4). The alterations in the HPA isoforms expression and GAG profile could be useful to detect cell kidney carcinomas and potentially target future therapeutic molecules.

Keywords: carcinoma, glycosaminoglycans, heparanase, isoforms, kidney

09.3- Role of the Microenvironment in the Treatment of Melanoma Cells with Doxorubicin

*Carla Abdo Brohem, Rafael Duarte Paes, Silvia Berlanga de Moraes Barros,
Silvia Stuchi Maria Engler*

1. FCF - USP, School of Pharmaceutical Sciences, Av. Professor Lineu Prestes, 580
Bloco 17 sala114. CEP 05508-900

In the past 50 years, melanoma incidence in developing countries grew faster than any other type of cancer, mainly due to the sun exposure. During tumor progression, the tumor cells are in contact with macromolecules of extracellular matrix (ECM), which mediates the adherence, migration, proliferation and gene expression of these cells. The melanoma is characterized by its high capacity of invasion, metastasis and chemoresistance to drugs. The tumor invasion and metastasis occurs when there are proteolytic degradation of basement membrane and ECM components by matrix metalloproteinases (MMPs). This work establishes the role of the microenvironment in the treatment of melanoma cells with doxorubicin, an antibiotic used as chemotherapy in solid tumors. The cytotoxicity, MMP-2 activity and p53 protein expression were evaluated when metastatic melanoma were cultured on type I collagen containing human dermal fibroblast (dermal equivalent culture), simulating *in vitro* the environment of tumor invasion. Fibroblast of human dermis (FF-287) were cultured in type I collagen, forming the dermal equivalent that serves as substrate for human melanoma cell line (SK-Mel-103) growth. The toxicity of Doxorubicin was tested by the Trypan blue exclusion assay. Zymography was performed to analyze MMP-2 activity, and Western Blotting to evaluate p53 protein expression. Cytotoxicity of Doxorubicin (0.7 mg/ml) is lower in the presence of the dermal equivalent (17%), when compared to the other conditions: uncoated substrate (75%) or seeded on type I collagen (67%) after 24 and 48 hours of treatment. In addition, inhibition of p53 protein activation and MMP-2 activity by doxorubicin are seen when melanomas are grown on the dermal equivalent compared to the other conditions. From these results we conclude that the dermal equivalent culture offers a protection to the melanoma cell when treated with doxorubicin, when compared to uncoated

substrate, showing the importance of the microenvironment in drugs screening tests. Support: FAPESP

Keywords: *doxorubicin, melanoma, microenvironment*

09.4- Heparanase Expression and Glycosaminoglycans Profile in Diffefrent Breast Cancer Cell Lines

*Lilian Zerbinatti de Oliveira¹, Maria Aparecida da Silva Pinhal^{1,2},
Helena Bonciani Nader¹*

1. UNIFESP, Universidade Federal de São Paulo, Rua Três de Maio, 100 - Vila Clementino - cep 04044-020 - São Paulo. 2. FMABC, Faculdade de Medicina do ABC, Avenida Lauro Gomes, 2000 - Vila Sacadura Cabral - cep 09060-870 - Santo André

The fact that heparan sulfate proteoglycans are so widely distributed is testament to the vital importance of these molecules in cell development and function. Remodeling of extracellular matrix following heparanase cleavage of heparin sulfate chains results both in liberation of glycosaminoglycan-anchored bioactive molecules fragments that modulate growth factor binding to their receptors (Sanderson et al., 2004). The amount of heparanase required for heparin sulfate remodelling appears to be of key importance as low levels of the enzyme enhances FGF2 binding and subsequent downstream activation of ERK/FAK signalling in human metastatic cells, whereas very high levels are inhibitory (Reiland et al., 2006). Heparanase-1 (HPA1) gene is formed by 40 kb localized at human chromosome 4 (4q21.3) (Vlodavsky I., et al. 1999). A second cDNA had recently been encoded for a new human isoform, named heparanase-2 or HPA2, encoded at chromosome 10q23-24 (Mackenzie et al. 2000). The main objective of this study was to correlate heparanase isoforms expression, glycosaminoglycans profile and breast tumor development. Heparanase isoforms expression were analyzed by RT-PCR and sulfated glycosaminoglycans were identified and quantified by agarose gel electrophoresis and densitometry using different breast cancer cell lines (MCF-7, MDA-MB-231 e SKBR-3) and a non tumoral breast cell line (MCF-10A). Confocal microscopy assay has shown that HPA1 cloned from MCF-7 presented a lysosomal localization. The results demonstrated that stable transfected HPA1 MCF-7 cells, that over expressed HPA1 not only changed GAG profile, but also decreased syndecan-1 proteoglycan synthesis and enhanced HPA2 expression. The data elucidated that there is a correlation between heparanase expression, sulfated glycosaminoglycans and proteoglycan synthesis in the breast cancer cell lines that possibly could explain different patterns of tumor development. Supported by FAPESP, CNPq and CAPES.

Keywords: *heparanase, heparan sulfate, proteoglycans, glycosaminoglycans, breast cancer*

09.5- Microarray Analysis of Differentially Expressed Genes in Tumor Breast Cells Treated with Laminin Derived Peptide C16

Emerson de Souza Santos, Vanessa Moraes Freitas, Ruy Gastaldoni Jaeger

1. ICB-USP, Instituto de Ciências Biomédicas - Universidade de São Paulo, Av Lineu Prestes, 1524, sala 405, Cidade Universitária, São Paulo/SP

Human breast cancer constitutes worldwide health care problem and represents 10% of all human malignant tumors. A complex pattern of genetic and epigenetic alterations is involved in the process of tumorigenesis, which often confers to transformed cells a higher proliferative potential, evasion to apoptosis, sustained angiogenesis, and capacity to invade the surrounding tissue and metastasize. Extracellular matrix molecules play important role influencing malignant behavior. Our Laboratory has been studying the involvement of laminin and its bioactive peptides in regulation of normal and neoplastic glandular cells. We have found that C16 (KAFDITYVRLKF), a peptide derived from laminin-111 gamma-1 chain, induces migration, invasion, and protease activity in salivary gland cells. This prompted us to study whether this peptide would regulate gene expression in breast gland cells. In this study, cells derived from breast tumors (MCF-7 and MDA-MB-231) were treated with C16. Cells treated with scrambled peptide (FKLRVYTIDFAK) served as control. After treatment for 24 hours, cells were collected for total RNA extraction, followed by cDNA and cRNA synthesis, and hybridization with microarray membranes. GEArray Expression Analysis Suite software was used to normalize and analyze the microarray data. Normalization was done using housekeeping genes (*GAPDH*, *B2M*, *HSP90AB1*, and *ACTB*). Preliminary results show that C16 regulates genes involved in cell cycle control, cell adhesion, signal transduction, cell death, invasion and metastasis in both MCF-7 and MDA-MB-231. Among these genes *HTATIP2*, a metastasis suppressor, was downregulated in both cell lines upon C16 treatment, while *CDK4* (cyclin-dependent kinase 4) expression was increased in both cells. *MMP2* (matrix metalloproteinase-2) expression, on the other hand, was upregulated in MDA-MB-231 but not in MCF-7 cells. Our data suggest that C16 induces differential expression of cancer-related genes in breast tumors. These genes may be associated with relevant biological effects induced by C16.

Keywords: *C16, LAMININ, BREAST, CANCER, MICROARRAY*

09.6- The Role of Anti-Inflammatory Protein Annexin A1 And Soluble Paracrine Factors on the Interactions Between Stromal and Tumor Cells

Thaís Santana Gastardelo¹, Flávia Cristina Rodrigues Lisoni², Eloiza Helena Tajara², Sonia Maria Oliani^{3,1}

1. UNIFESP, Pos-graduation Morphology – Federal University of São Paulo, Rua Botucatu, 740. CEP:04023-900. São Paulo, SP.. 2. FAMERP, Department of Molecular Biology - School of Medicine, Av. Brigadeiro Faria Lima, 5416. CEP:15090-000. São José do Rio Preto, SP. 3. IBILCE-UNESP, Department of Biology – São Paulo State University, Rua Cristóvão Colombo, 2265. CEP:15054000. São José do Rio Preto, SP.

Several evidences have showed that the development and progression of cancer depends on the interactions between stromal and tumor cells. Recent studies have demonstrated that the anti-inflammatory protein annexin A1 (ANXA1) is related to the control of tumor cell growth. In the present study we investigated the growth curve of Hep-2 cells (derived from human larynx epidermoid carcinoma) and fibroblasts (derived from tumor stromal) after treatment with the peptide Ac2-26 (N-terminal sequence of ANXA1) and soluble paracrine factors, produced by stromal and neoplastic cells. Besides, we analysed the ANXA1 expression in the Hep-2 cells and fibroblasts, by immunocytochemistry and PCR real time. In order to investigate the effect of soluble factors produced by cells, Hep-2 cells received the conditioned medium from tumor stromal fibroblast and fibroblasts received the conditioned medium from Hep-2 cell. Hep-2 cells and fibroblasts were maintained in control medium (C), in control medium treated with the peptide (C+P), in conditioned medium (CM) and in conditioned medium treated with the peptide (CM+P), at 6, 24, 72, 120 and 168 hours. Both CM and peptide treatments resulted in decreased on Hep2 and fibroblast proliferation, suggesting that factors produced by both cells and the ANXA1 interfere in cell growth. The decreased of Hep-2 growth was observed at 72 and 120h after the treatments (C+P), (CM) and (CM+P). Fibroblast growth was inhibited by CM and peptide treatments during all 168 hours. The ANXA1 protein expression, analysed by immunocytochemistry, was increased in Hep-2 at 72h after CM treatment. Fibroblast showed high ANXA protein expression at 72h after treatment with CM+P. The ANXA1 gene expression, analysed by PCR, was increased in Hep-2 cells at 6h after peptide and CM treatments. In contrast, fibroblasts showed reduced ANXA1 gene expression at 6h under all conditions. These data stress the important role of ANXA1 and soluble paracrine factors on the regulation of stromal and tumor cell proliferation. The importance of the ANXA1 is emerging and is likely to contribute to our understanding of the link among inflammation, hyperproliferation and carcinogenesis. Their roles in molecular pathways as well as their clinical implications, are just beginning to emerge in the literature. However, more information on the subject and prospective studies which deal with diagnosis, prognosis and treatment are still needed.

Keywords: *Annexin A1, cancer, fibroblast, Hep-2 cell, soluble paracrine factors*

09.7- Structural and Molecular Disorganization of Collagen V is Associated with Tumoral Growth in Non-Small Cell Lung Cancer

Paola da Costa Souza, Fabrizio Rizzardi, Gustavo Noleto, Marcelo Atanazio, Osmar Bianchi, Edwin Roger Parra, Walcy Teodoro, Ana Paula Velosa, Solange Carrasco, Sandra Fernezlian, Alexandre Muxfeldt Ab'Saber, Teresa Takagaki, Cláudia Goldenstein Schainberg, Natalino Hajime Yoshinari, Vera Luiza Capelozzi, Leila Antonangelo

1. FMUSP, Faculdade de Medicina da Universidade de São Paulo, AV Dr Arnaldo 455, sala 1143, CEP 01296-903, São Paulo, SP, Brazil

Collagen V shows promise as an inducer of the death response via caspases. Remodeling of the microenvironment by collagen V, tumoral/vascular apoptosis and the immune response were evaluated, based on the prognosis of 65 patients with surgically excised non-small cell lung cancer. Immunofluorescence, immunohistochemistry, morphometry, tridimensional reconstruction and a real-time polymerase chain reaction were used to evaluate the amount,

structure and molecular chains of collagen V, tumoral and vascular apoptosis, immune cells and microvessel density. The impact of these markers was tested on follow-up until death from recurrent lung cancer occurred. A decreased and abnormal synthesis of collagen V was found to lead to increased angiogenesis due to a low endothelial death rate and a low immune response. A Cox model analysis, controlled for the lymph node stage, demonstrated that only collagen V and vascular apoptosis variables were significantly associated with survival time. A point at the median for collagen V and vascular apoptosis divided patients into two groups, each with a distinctive prognosis. Those with a collagen V higher than 9.40 % and vascular apoptosis higher than 1.09 % had a low risk of death (0.27 and 0.41, respectively), compared to those with a collagen V lower than 9.40% and vascular apoptosis lower than 1.09%. Collagen V and vascular apoptosis in resected non-small cell lung cancer was strongly related to the prognosis, suggesting that strategies aimed at preventing low collagen V synthesis, or local responses to low vascular apoptosis, may have a greater impact in lung cancer treatment.

Keywords: *Apoptosis, Caspase 9, Collagen V, Lung Cancer, Survival*

09.8- Study of Galectins and Mast Cells in the Experimental Gastric Carcinogenesis in Rats

Cassia Fernanda Estofolete, Ana Cláudia Polli Lopes, Cristiane Damas Gil

1. FAMERP, São José do Rio Preto School of Medicine, Av. Brigadeiro Faria Lima, 5416, CEP 15090-000, São José do Rio Preto-SP, Brazil

Galectins are a family of proteins defined by a conserved carbohydrate-recognition domain with affinity for beta-galactosides. In cancer biology, galectin-1 (Gal-1) and -3 (Gal-3) can play crucial roles in tumor cell-cell or cell-matrix interactions through their binding activities to the tumor cell surface carbohydrate. The mast cells (MCs) represent one of main immune cell types implicated in tumor-associated inflammation and present Gal-1 and -3 expressions in their cytoplasmic granules that can be modulated during inflammatory response. In this study, MC distribution and Gal-1 and -3 expressions were evaluated in an experimental gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats with chemical denervation of the stomach with benzalkonium chloride. Four experimental groups were evaluated: non denervated (group I) and denervated (group II) without lesions; non denervated (group III) and denervated (group IV) with lesions. Histological sections of the pyloric region were stained with Toluidine Blue for histopathological analysis and with Alcian Blue-Safranin (AB-SAF) for heterogeneity and distribution of MCs. For immunohistochemical study of galectins, sections of the stomach were incubated with monoclonal mouse anti-Gal-1 and polyclonal rabbit anti-Gal-3. MNNG treatment provoked the development of benign and malignant gastric lesions with stromal tissue enriched by inflammatory cells (MCs, neutrophils, plasm cells and lymphocytes). Mucosal MCs (AB positives) were observed mainly in animals of groups I and II, whereas the phenotypes AB and AB-SAF positives were detected in the gastric lesions of animals from groups III and IV. The increase in the number of MCs in the gastric tissues was associated with the development of lesions induced by MNNG treatment, compared to groups I and II. Gal-1 expression was detected in MCs of all experimental groups and in ECM of gastric

lesions. Gal-3 was strongly positive in MCs, leukocytes and ECM of peritumoral lesions. These results showed a strong association among the density of MCs, Gal-1 and -3 expressions and the development of gastric neoplasms induced by MNNG in the stomach of denervated or not denervated rats. Then, the study of MCs and galectins as a target of new therapies in the process of tumorigenesis and their future clinical application must be considered. Supported by FAPESP.

Keywords: *galectin-1, galectin-3, gastric lesions, myenteric denervation, immunohistochemistry*

09.9- Sulfated Polysaccharides Isolated from Marine Invertebrates Inhibit Prostate Cancer Cell Proliferation and Interaction with Endothelial Cells in Vitro

Camila Castro Figueiredo¹, Viviane Wallestein Mignone¹, Aline Oliveira Silva¹, Tercia Rodrigues Alves¹, Paulo Antônio de Souza Mourão², Verônica Morandi¹

1. UERJ, Universidade do Estado do Rio de Janeiro, Departamento de Biologia Celular, LabAngio, PHLC, sala 203. 2. UFRJ, Universidade Federal do Rio de Janeiro, Instituto de Bioquímica Médica, CCS, Ilha do Fundão

To form metastases tumor cells must complete a series of steps, including the invasion of normal tissues and intravasation through endothelial cells in direction of the bloodstream. Once in the circulation, tumor cells must be able to adhere and migrate across the endothelium (extravasation). After adherence to endothelial cells and subsequent endothelial retraction, metastatic tumor cells must adhere to elements of the subendothelial basement membrane, and migrate into the subendothelial stroma to grow at this new site. Interactions between endothelial selectins and selectin ligands expressed on tumor cells have been implicated in the binding of circulating metastatic cancer cells to the vascular endothelium during extravasation. Cancer cells frequently exhibit abnormal glycosylation patterns, resulting in the synthesis and expression of altered carbohydrate determinants including sialyl Lewis^x and sialyl Lewis^x, that are recognized by endothelial selectins. Recent studies suggest that sulfated polysaccharides could inhibit the metastasis and proliferation of tumor cells by binding to growth factors and cell adhesion molecules. In this work, we studied the effect of the sulfated polysaccharides isolated from sea urchin (*Lytechinus variegatus*, *Strongylocentrotus pallidus*, *Strongylocentrotus franciscanus*, *Glyptocidaris crenularis* and *Echinometra lucunter*) and from sea cucumber (*Ludwigothurea grisea*) in the proliferation and interaction of prostate cancer cell with endothelial cells (HUVECs) and subendothelial extracellular matrix. We observed that the sulfated polysaccharides (100 µg/ml) inhibited prostate tumor cells adhesion and transmigration through TNF-α activated endothelial cells. However, only the polysaccharide isolated from *L. variegatus* was able to inhibit the adhesion of tumor cells to the native subendothelial extracellular matrix, by binding directly to this substrate. All polysaccharides, with exception of that isolated from *G. crenularis*, inhibited prostate tumor cell proliferation after 72 hours. The polysaccharide isolated from *L. variegatus* was the most effective growth inhibitor since the effect was visible in the first 24 hours (58% of inhibition) and was maintained until 72 hours (50% of inhibition). These

results suggest that these polysaccharides, mainly that isolated from *L. variegatus*, may be potential candidates for an anti-metastatic drug. Supported by CNPq, Capes and Faperj.

Keywords: Adhesion, Endothelial Cells, Extracellular Matrix, Polysaccharides, Tumor Cells

09.10- The RECK B Isoform is Downregulated by Simvastatin in Human Melanoma Cells

Fernanda Augusta de Lima Barbosa¹, Marina Trombetta Lima², Thiago Jacomasso¹, Mari Cleide Sogayar², Glaucia Regina Martinez¹, Sheila Maria B. Winnischofer¹

1. UFPR, Universidade Federal do Paraná, CP: 19046, CEP:81531-990. 2. USP, Universidade de São Paulo, CP:26077, CEP: 05513-970

RECK encodes a membrane-anchored protein which suppresses invasion/metastasis by negatively regulating matrix metalloproteinases, particularly, MMP-2, MMP-9 and MT1-MMP. A positive correlation has been found between the relative abundance of RECK expression in tumor samples and better prognosis for patients with gastric, lung, pancreatic and colorectal cancers. In addition to downregulating cholesterol levels, statins exert other effects, which include anti-inflammatory and anti-proliferative actions. In particular, it has been described that Simvastatin (SIM) is able to inhibit melanoma cells growth and invasion. However, the molecular mechanisms underlying these delayed drug-induced effects are still unclear. Here, we evaluated the viability of human melanoma cell lines (SK-Mel28 and MeWo), through crystal violet staining, upon treatment with SIM at different concentrations (0.1, 0.5, 1, 5 and 10 $\mu\text{mol.L}^{-1}$) for 48 and 72h. For SK-Mel28 cells, we observed a 20% reduction in viability upon treatment with SIM for 48h. After 72h, we observed 45 and 51% reduction in cell viability when these cells were treated with SIM at 5 and 10 $\mu\text{mol.L}^{-1}$, respectively. On the other hand, SIM had no effect in viability of MeWo cells upon treatment for 48h. Only after 72h, we observed 20 and 28% reduction in viability when MeWo cells were treated with SIM at 5 and 10 $\mu\text{mol.L}^{-1}$, respectively. In order to evaluate the molecular mechanism involved in the anti-proliferative effects of SIM in melanoma cells, we analyzed the mRNA expression levels of the RECK gene (canonical form and alternative isoforms, namely: RECK B, RECK C, RECK D and RECK I) in melanoma cells treated with SIM at 1 and 5 $\mu\text{mol.L}^{-1}$ for 72h. The expression profiles of these genes were investigated through quantitative real time RT-PCR assays. Preliminary results indicate that the mRNA levels of the RECK B isoform is significantly reduced in SK-Mel28 cells upon treatment with SIM in a dose-dependent manner. The RECK B isoform showed a tendency to be downregulated also in MeWo cells treated with SIM. On the other hand, the mRNA levels of the RECK canonical form and of other isoforms (RECK C, D and I) were not altered upon treatment with this drug. Our results suggest that differently from the canonical form, the RECK B isoform may play an important role in the molecular signaling induced by SIM in melanoma cells. Support: FAPESP, CAPES, CNPq, FINEP, Redoxoma.

Keywords: Melanoma, MMPs, RECK

09.11- Laminin-Derived Peptide Ag73 Increases Invadopodia Activity in a Cell Line Derived from Adenoid Cystic Carcinoma

Camila Fernandes Nascimento, Vanessa Moraes Freitas, Ruy Gastaldoni Jaeger

1. ICB - USP, Instituto de Ciências Biomédicas - Universidade de São Paulo, Av. Prof. Lineu Prestes, 1524 - sala 405 CEP 05508-900 São Paulo SP

Our laboratory studies effects of laminin and its derived peptides in tumor biology. We demonstrated that the peptide AG73 (RKRLQVQLSIRT, LG4 domain of laminin alpha1 chain) is involved in migration, invasion and protease of a cell line (CAC2) derived from adenoid cystic carcinoma. This tumor is a malignant salivary gland neoplasm with recurrence and metastasis long time after initial therapy. Metastatic tumor cells that actively migrate and invade surrounding tissues rely on invadopodia to degrade extracellular matrix (ECM) barriers. Invadopodia are actin-rich membrane protrusions that localize enzymes required for ECM degradation. These protrusions contain actin, cortactin and membrane type 1 matrix metalloproteinase (MT1-MMP) superimposed to areas of digested matrix. We have already characterized invadopodia in CAC2 cells (Nascimento et al., 2009). Here we studied whether the peptide AG73 would increase invadopodia activity of CAC2 cells. Cells were treated with AG73. Control cells were treated with scrambled peptide (AG73SX). Treated and control samples were subjected to fluorescent-substrate degradation assay, to assess in situ protease activity of CAC2 cells. Cells were cultured overnight on gelatin-FITC, followed by fixation and labeling to actin. In this assay, digestion spots in fluorescent substrate appear as black areas in green background. On the other hand, actin staining outlines cell shape and identifies protrusion (invadopodia) superimposed to digested areas. Samples were studied by fluorescence microscopy. Invadopodia counting showed that AG73 significantly increased in situ protease activity of CAC2 cells compared to controls. Our preliminary results suggested that laminin-derived peptide AG73 enhanced invadopodia formation and protease activity of CAC2 cells. Support: FAPESP(2006/57079-4 and 2008/57103-8) e CNPq (304868/2006-0 and 470622/2007-5).

Keywords: *adenoid cystic carcinoma, AG73, invadopodia, laminin*

09.12- Laminin-Derived Peptide Ag73 Regulates Migration, Invasion and Protease Activity of Human Oral Squamous Cell Carcinoma Cells Through Syndecan-1 and Beta1 Integrin

Adriane Sousa de Siqueira¹, Letícia Nogueira da Gama de Souza^{1,3}, João de Jesus Viana Pinheiro^{1,2}, Ruy Gastaldoni Jaeger¹

1. ICB-USP, Institute of Biomedical Sciences - University of Sao Paulo, Av. Prof. Lineu Prestes, 1524, sala 405 - Cidade Universitária, São Paulo, SP. 2. UFPA, School of Dentistry - Federal University of Para, Av. Augusto Corrêa, n. 1 - Guamá, Belém, PA. 3. CCS-UFES, Health Sciences Center- Federal University of Espirito Santo, Av. Fernando Ferrari, 514- Goiabeiras, Vitória, ES

Oral squamous cell carcinoma is a prevalent head and neck tumor, related to high mortality rates. Its growth and invasiveness is stimulated by interactions with extracellular matrix and basement membrane, which prominently expresses laminin. This molecule regulates tumor biology and harbors cryptic sites with important roles upon release by proteolytic enzymes such as matrix metalloproteinases (MMPs). MMP-mediated breakdown of basement membrane is a critical step in tumor progression that involves laminin cleavage and release of peptides which may influence carcinoma's behavior. Our Laboratory studies the effects of laminin-derived peptides in tumor biology. Here we analyzed the role played by AG73 (RKRLQVQLSIRT, alpha1 chain) on migration, invasion and protease activity of a cell line (OSCC) from human oral squamous cell carcinoma. Laminin alpha 1 chain and MMP9 were immunolocalized in human oral squamous cell carcinoma cells *in vivo* and *in vitro*. AG73 increased migratory activity of OSCC cells, as shown by monolayer wound assay and migration assay. This peptide also stimulated cell invasion in chemotaxis chambers coated with Matrigel. Invasion depends on protease activity. OSCC cells cultured on AG73 showed a dose-dependent increase of MMP9 secretion. We searched for AG73 receptors regulating activities in this cell line. OSCC cells grown on AG73 exhibited colocalization of syndecan-1 and beta1 integrin. siRNA knockdown of syndecan-1 and beta1 integrin decreased AG73-dependent migration and invasion of OSCC cells. Our results suggest that syndecan-1 and beta1 integrin signaling downstream of AG73 regulate migration, invasion and MMP production by OSCC cells. Support: FAPESP (06/57079-4, 07/51950-8), CNPq (470622/2007-5).

Keywords: *Oral squamous cell carcinoma, Laminin, Matrix metalloproteinases, Syndecan-1, Beta1 integrin*

09.13- Regulation of Mmps Expression by HPV16 Oncoproteins in Primary Epithelial Cultures

Laura Beatriz da Silva Cardeal^{1,2}, Enrique Boccardo², Tatiana Rabachini², Luisa Lina Villa², Silvy Stuchi Maria-Engler¹

1. FCF/USP -SP, Universidade de São Paulo Faculdade Ciências Farmacêuticas, Av. Lineu Prestes, 580, Bloco 17, sl 114, São Paulo, SP, CEP05508000. 2. ILPC/SP, Instituto Ludwig de Pesquisa Sobre o Câncer LTDA, Hospital Alemão Oswaldo Cruz, Rua João Julião 245, 1º Andar, Paraíso, São Paulo, SP

Cervical cancer is etiologically associated to high-risk human papillomavirus (HPV) infection. Matrix metalloproteinases (MMPs) -2, -9, and MT1-MMP are required for cervical carcinoma progression, but is still unclear whether the macrophages rather than keratinocytes contribute to their secretion. Moreover, a counterbalancing among MMPs and their regulators, such as TIMPs and RECK, is necessary to prevent invasion. In order to study the effect of HPV oncogenes on MMPs expression, primary human keratinocytes (PHKs) were infected with recombinant retroviruses expressing wild-type HPV16 E6 and/or E7 oncogenes and were used to seed epithelial cultures. Quantitative real-time PCR (Q-PCR), western blot, zymography and gelatinolytic assay were used to determine the expression level and activity of MMP-2, MMP-9, MT1-MMP and their inhibitors RECK and TIMP-2. We observed that,

when individually expressed, HPV16 oncoproteins exert a differential effect on MMP-9. While MMP-9 activity was down-regulated in cultures expressing HPV16 E7 oncogene, its activity was up-regulated in HPV16 E6-expressing cells. On the other hand, cultures expressing both E6 and E7 exhibited MMP-9 levels similar to those observed in PHKs infected with the empty vector (pLXSN). Interestingly, these cells expressed lower RECK levels than PHKs transduced with the empty vector or with HPV16 E6 or E7. Furthermore, we observed that expression of HPV16 E6 and/or E7 proteins do not affect MMP-2, MT1-MMP and TIMP-2 levels and/or activity. However, the gelatinolytic assay showed that cells expressing HPV16 E6 exhibit a different degradation pattern when compared to the others cultures. Finally, we show that the gelatinolytic degradation depends on MMPs activity since it is decreased by a MMPs pan inhibitor (Ilomastat). Taken together, our results demonstrate that HPV16E6E7 presence down-regulates RECK protein levels, leading us to suggest that the both E6 and E7 oncoproteins contribute to modulate the matrix degradation in cervical carcinoma. Financial Support: Fapesp, CNPq, FINEP, PRP-USP and LICR.

Keywords: *Metalloproteinases, RECK gene, HPV, Cervical carcinoma*

09.14- RECK-Mediated Inhibition of Glioma Migration and Invasion

Renato Ramos Massaro¹, Tatiana Caroline S. Corrêa¹, Carla Abdo Brohem¹, Sebastião Roberto Taboga², Marcelo Lazzaron Lamers³, Marinilce Fagundes Santos³, Silvy Stuchi Maria-Engler¹

1. FCF - USP, Faculdade de Ciências Farmacêuticas - Universidade de São P, Av. Prof. Lineu Prestes, 580 - Butantã - São Paulo – SP. 2. IBILCE - UNESP, Instituto de Biologia - Universidade Estadual de São Paulo, Rua Cristovão Colombo, 2265. São José do Rio Preto – SP. 3. ICB - USP, Instituto de Ciências Biomédicas - Universidade de São Paulo, Av. Pfof Lineu Prestes, 1524 - sala 1524 - Butantã - São Paulo - SP

RECK is an important tumor suppressor gene in which anti-tumoral activity has traditionally been associated to its inhibitory effects on MMPs, regulating at least MMP-2, MMP-9 and MT1-MMP. Elevated MMP activity due to lack of functional RECK compromises tissue integrity. RECK levels have been shown to be decreased in progression of glioblastoma from less invasive grade II gliomas to very invasive human glioblastoma multiforms (GBMs). Due to the inverse correlation between RECK expression and glioma invasiveness, we hypothesized that RECK expression would compromise the invasive behavior of these gliomas. We therefore conducted this study to explore forced RECK expression in the highly invasive T98G human GBM cell line, which displays high levels of both MMP-2 and -9. Expression levels as well as protein levels of RECK, MMP-2, MMP-9 and MT1-MMP were assessed by qPCR and immunoblotting in T98G/RECK+ cells (stable transfection of T98G overexpressing RECK). The invasion and migration capacity of this GBM cell line expressing high levels of RECK was shown to be inhibited in transwell and wound assays. Dramatic cytoskeleton modifications were observed in the T98G/RECK+ cells, when compared to control cells, such as the abundance of stress fibers (contractile actin-myosin II bundles) and reduction of lamellipodia. T98G/RECK+ cells also displayed phosphorylated focal adhesion kinase (P-FAK) in mature focal adhesions associated with

stress fibers; whereas P-FAK in control cells was mostly associated with immature focal complexes. Interestingly, the RECK protein was predominantly localized to the leading edge of migrating cells, associated with membrane ruffles. Surprisingly, in this glioma model, the overexpression of RECK had no detectable effects on MMP-2 and -9 as predicted; no alterations were observed at the mRNA level (qPCR), at the protein level (immunoblotting) nor in protein activity (gelatin zymography). Unexpectedly, introduced expression of RECK effectively inhibited the invasive process through rearrangement of actin filaments, promoting a decrease in migratory ability. This work has associated RECK tumor suppressor activity with the inhibition of motility and invasion in this GBM model, which are two glioma characteristics responsible for the inefficiency of current available treatments.

Keywords: *RECK, migration, invasion, glioma*

09.15- Apoptotic Effect of a Plant Kallikrein Inhibitor on Prostate Cell Lines

Joana Gasperazzo Ferreira, Cláudia Alessandra Andrade de Paula, Paula Malloy Mota Diniz, Adriana Miti Nakahata, Misako Uemura Sampaio, Maria Luiza Vilela Oliva

1. UNIFESP, UNIVERSIDADE FEDERAL DE SÃO PAULO, Rua 3 de maio, n. 100, Vila Clementino, São Paulo, SP, Brasil

Prostate cancer occurs with high rates of incidence, mortality and invasion from the primary tumor. Tissue kallikrein hK3 (PSA) and integrins are directly related to prostate neoplasia, interfering in cell adhesion, differentiation and cellular proliferation. Compounds that interfere in these processes are targeted for investigation. In this work, using prostate cancer cellular lines PC3 and DU145, we studied the effect of recombinant kallikrein inhibitor (rBbKI) and its modified form (rBbKm), in which the sequence containing the signal motif sequence RGD of the inhibitor BrTI from *Baunina rufa* was inserted. rBbKIm inhibited cell proliferation of PC3 (70%, 50 and μM , 48 h) and of DU145 (60%, 100 and μM , 48 h) but did not affect the proliferation of fibroblast normal cell line (100 and μM , 48h). rBbKIm affects cell cycle of PC3, increasing apoptosis and decreasing cell G2 fase (mitosis). In cell adhesion, rBbKIm (25 and μM) containing the adhesion motif RGD, inhibited approximately 30% of the PC3 adhesion on fibronectin, whereas rBbKI inhibition was 20%. The confocal microscopy analysis showed that both rBbKI and rBbKIm interact with cell membrane. Previous studies showed that rBbKIm induces nitric oxide (NO) release upon inducible nitric oxide synthase (iNOS) activation. This property is under investigation in order to clarify if the apoptotic effect of rBbKIm is correlated to its mechanism of action. Supported by CNPq, CAPES, FAPESP, FADA/FAP and UNIFESP.

Keywords: *kallikrein inhibitor, Prostate, Cancer, PC3, DU145*

09.16- Head and Neck Cancer Progression to Metastatic State Involves Cathepsin B and Heparan Sulfate Glycosaminoglycans Levels Modifications

Fábio Dupart Nascimento¹, Nilana M. T. Barros², Tarsis G. Ferreira², Helena B. Nader², Ivarne L. S. Tersariol³

1. UNIBAN, Universidade Bandeirante, Av. Maria Candida 1813, bloco G 6 andar - CEP: 02071-013. 2. UNIFESP, Universidade Federal de São Paulo, Rua três de maio 100, 4 andar. CEP:04044-020. 3. UMC, Universidade de Mogi das Cruzes, Av. Candido Xavier de Almeida e Souza 200. CEP: 08780

Epithelial to mesenchymal transformation is the first manifestations occurring during the transformation of epithelial cells into malignant carcinoma. As cells lose cell-cell contact and cell-extracellular matrix interaction, new growth, motility, ECM degradation are generated during metastasis. Close correlations have been observed between Cathepsin B and Heparan Sulfate levels at the cell surface of squamous cell carcinoma. Oral cancer is one of the 10 most frequently occurring cancers worldwide. The aim of this study is to investigate the involvement of Cathepsin B and Heparan Sulfate glycosaminoglycans during the cell differentiation process. Cathepsin B and Heparan Sulfate amounts, localization, activity and gene expression between a primary human hypopharynx carcinoma cells, line UMSCC–22a, and its neck metastatic cells, clone UMSCC-22b has been analyzed. The results obtained by qPCR analysis showed that the metastatic clone UMSCC-22b have an increased expression of Cathepsin B mRNA comparing with the primary tumor cell line. Gene expression levels of Heparan Sulfate proteoglycan (Syndecan) and Chondroitin Sulfate proteoglycan (Versican) were also analyzed by qPCR showing significant differences between both cell lines. On direct glycosaminoglycans analyzes Heparan Sulfate levels of metastatic cells decrease 5.6-fold and Chondroitin Sulfate was increased 8-fold in relation of primary tumor cells at cell surface following the data obtained on proteoglycans mRNA study. Also, both immunocytochemistry analysis by flow cytometry and confocal microscopy images showed that the intracellular amount of cathepsin B protein was increased in the metastatic cells. More, the enzymatic determination of cathepsin B by using the fluorogenic Z-FR-MCA substrate showed that the metastatic clone UMSCC–22b exhibited 6-fold more cathepsin B activity than its primary tumor cells UMSCC–22a. Take together, these results show that the primary tumor cells UMSCC-22a undergo a epithelial to mesenchymal transformation conversion to the migratory metastatic UMSCC-22b phenotype. *Supported By: FAPESP, CNPq and CAPES*

Keywords: *Cancer, Cathepsin B, Glycosaminoglycans, Heparan Sulfate, Chondroitin Sulfate*

09.17- Laminin Chains Alpha 1 and Gamma 2 are Cleaved in Oral Human Squamous Cell Carcinoma, Generating Fragments with Relevant Biological Role

João de Jesus Viana Pinheiro^{2,1}, Luciana Rodrigues Lobo¹, Ruy Gastaldoni Jaeger¹

1. USP, University of Sao Paulo, Institute of Biomedical Sciences, Av. Prof. Lineu Prestes, 1524 Ed. Biomedicas 1, sala 302. Sao Paulo, SP. 2. UFPA, Universidade Federal do Pará, Rua Augusto Corrêa, 01 - Guamá. CEP 66075-110. Caixa postal 479.

Squamous cell carcinoma represents 95% of malignant oral neoplasms and constitutes an important health problem. Microenvironment of carcinoma is formed not only by neoplastic cells, but also by surrounding stroma. Therefore, its progression requires tumor cell interactions with extracellular matrix (ECM). In epithelia, cells form a specialized ECM sheet-like structure, the basement membrane, composed by laminin, type IV collagen, nidogen and perlecan. Laminins are heterotrimeric glycoproteins prominently expressed in basement membranes that promote cell adhesion, migration, growth and differentiation. Here we studied expression of different laminin chains, such as $\alpha 1$, $\alpha 3$, $\beta 1$, and $\gamma 2$ in human oral squamous cell carcinoma. Fifty-five cases of this neoplasm were studied by immunohistochemistry. Sections from formalin-fixed paraffin-embedded tissues were subjected to EnVision System. Antigen retrieval was carried out with citrate buffer (pH 6.0) in Pascal chamber for 30 seconds. Laminin chains $\alpha 1$, $\alpha 3$, $\beta 1$, and $\gamma 2$ were immune localized in human oral squamous cell carcinoma in vivo. It is known that laminin undergoes MMP processing, generating small fragments that can influence cell behavior. To address whether laminin from oral squamous cell carcinoma would release such bioactive fragments, tumor lysates were electrophoresed and immunoblotted with antibodies against different laminin chains. Samples from oral squamous cell carcinoma were compared to tissue extracts from human oral mucosa. Purified laminins 111(former laminin-1) and 332 (former laminin-5) run in the same gels served as positive controls. Immunoblot showed laminin chains $\alpha 1$, $\alpha 3$, $\beta 1$ and $\gamma 2$ in both oral squamous cell carcinoma and oral mucosa. Furthermore, carcinoma samples showed cleavage of $\alpha 1$ and $\gamma 2$ chains. Molecular weight of $\alpha 1$ fragment (~50kDa) was consistent with laminin-111 E3 domain. Molecular weight of $\gamma 2$ product (~80kDa) strongly suggested laminin $\gamma 2x$ fragment from laminin-332. It is widely known that both E3 and $\gamma 2x$ exhibit important biological effects in neoplastic cells. Our preliminary results indicate that laminin chains $\alpha 1$ and $\gamma 2$ undergo cleavage in human squamous cell carcinoma, generating fragments with relevant biological role.

Keywords: *Extracellular matrix, Laminin, Oral human squamous cell carcinoma*

09.18- Peptide A3-10, Derived From Laminin-332 Modulates Migration of Human Adenoid Cystic Carcinoma Cells

Karen Steponavicius Piedade Cruz, Adriane Sousa de Siqueira, Ruy Gastaldoni Jaeger

1. ICB-USP, 1Departament of Cell and Developmental Biology, AV. Lineu Prestes, 1524 sala 405

Adenoid cystic carcinoma is a frequently occurring malignant salivary gland neoplasm, with high level of recurrence and distant metastasis. This tumor prominently expresses extracellular matrix proteins. One of these proteins is laminin, a glycoprotein with important biological roles, including cellular adhesion, migration, differentiation, proliferation, tumor metastasis, and protease secretion. Our laboratory studies the roles of laminins and their bioactive peptides in tumor biology. The aim of this study was to analyze the effects of A3-10, a peptide derived from laminin-332 (also known as laminin-5), on migration and invasion in an adenoid cystic carcinoma cell line (CAC2 cells). A3-10 migration assays were carried out using a Neuro Probe chemotaxis chamber with porous membrane (8µm pore size), containing different A3-10 concentrations (10-100 µg/well). Same concentrations of scrambled peptide (A3-10SX) were used as controls. Results showed that A3-10 promoted a dose-dependent increase in migration rate of CAC2 cells. Invasion activity was assessed in Boyden chamber with membranes coated with Matrigel, using different peptide concentrations. No significant differences were found between invasion rates in A3-10 group compared to control A3-10SX. Our data suggest that A3-10 peptide promote migration, but not invasion, of human adenoid cystic carcinoma cells.

Keywords: *Adenoid Cystic Carcinoma, Extracellular Matrix, laminin-332*

09.19- The Role Played by Adamts-1 on Migration and Invasion of Malignant Mammary Gland Cells

Vanessa Moraes Freitas, Jonatas Bussador do Amaral, Ludimila Parra Mariano, Fabio Siviero, Gláucia Maria Machado-Santelli

1. ICB-USP, USP-Instituto de Ciências Biomédicas, Av. Prof. Lineu Prestes, 1524, 3 andar sala 307

ADAMTS-1 (a disintegrin and metalloprotease with thrombospondin motifs) is a member of the ADAMTS family of metalloproteases, and related to cancer progression. In spite of ADAMTS-1 biological relevance, mechanisms underlying its involvement in tumor biology remain elusive. ADAMTS-1 is downregulated in human breast carcinoma. Here we studied role played by ADAMTS-1 in migration and invasion of a malignant mammary cell line (MDA-MB-231). Cells had ADAMTS-1 silenced by either shRNA-GFP or siRNA. MDA-MB-231 cells transfected with scrambled oligos served as controls. Immunoblot confirmed silencing efficiency. Migration was investigated by time-lapse microscopy. Images (5 min interval) were recorded sequentially (4 hours). Tracking measurements yielded length (µm) and speed (µm/hour) of single cells. Silencing of ADAMTS-1 significantly increased migration of MDA-MB-231 cells. Knockdown of ADAMTS-1 also increased invasion of MDA-MB-231 cells in Boyden chambers coated with Matrigel. Silencing of ADAMTS-1 enhanced protease activity, as shown by zymography. Migration, invasion and protease activity are essential for tumor progression and metastasis. Metastatic cells rely on invadopodia to degrade and invade extracellular matrix (ECM). Invadopodia are finger-like membrane protrusions with enzymes required for ECM degradation. These protrusions contain cortactin and MT1-MMP superimposed to areas of digested matrix. Invadopodia proteins were studied by immunoblot on MDA-MB-231 cells with reduced expression of

ADAMTS-1. Silencing of ADAMTS-1 increased cortactin and MT1-MMP expression. We investigated role played by ADAMTS-1 on invadopodia formation and protease activity of MDA-MB-231 cells. These cells were transfected with an shRNA-GFP vector to knockdown ADAMTS-1. Control cells expressed non-silencing GFP vector. Treated and control cells were grown on fluorescent gelatin (gelatin-Alexa 647) overnight. In this assay, digested gelatin appears as black spots on fluorescent matrix. After growing overnight on fluorescent gelatin, MDA-MB-231 cells were fixed and stained to cortactin. Cells with silenced ADAMTS-1 exhibited expression of cortactin and increased number of digestion spots compared to controls. Our results suggested that ADAMTS-1 plays a role in migration, invasion and protease activity of MDA-MB-231 cells. Support: FAPESP (2006/54963-0), and CNPq (470779/2007-1).

Keywords: *ADAMTS-1, breast cancer cell lines, invasion, migration, proteases*

09.20- Collagen Type I Treatment Inhibits Grow of Tumor in Bearing Mice Melanoma and Breast Adenocarcinoma Activating Caspase-3.

*Vanessa Martins, Adilson Kleber Ferreira, Danilo Fernando de Oliveira Inada,
Ricardo Alexandre Azevedo, Durvanei Augusto Maria*

1. IBUT, Biochemistry and Biophysics Lab., Institute Butantan, AV. Vital Brazil, 1500

Introduction: Collagen type I is a prevalent component of the stromal extracellular matrix; its expression being spatially and temporally regulated during mammary ductal formation and dermal differentiation. The primary function of collagen fibers is to add strength to the connective tissue can have a stimulatory or inhibitory effect on cell proliferation and organized fibrillar structure inhibits normal and malignant cell proliferation. **Objective:** Evaluated the effects potential inhibits proliferative response and apoptosis by type I collagen in mice bearing B16F10 melanoma and adenocarcinoma of Ehrlich's tumor. **Methods and Results:** Were determined the inhibitory concentrations (IC50%) of collagen type I in B16F10 melanoma, breast adenocarcinoma of the Ehrlich and normal fibroblasts by the colorimetric method - MTT. Activity of collagen in induced cell death pathway caspase-3 was tested by enzymatic-fluorogenic method. The inhibitory concentrations (IC50%) were 18.6 mg for B16F10 melanoma and 49.9 mg for breast adenocarcinoma. Microscopic analysis of tumor cells showed multicellular aggregation and lost adhesion in extracellular matrix. The treatment by collagen o showed cytotoxic effects and morphologic changes but proliferation cellular and sites of collagen by fibroblasts dermal. The collagen type I had significant activity as a substrate for active site specific of proteolytic cleavage of caspase 3. The mice C57BL/6J and Balb-c were implanted with 5x10⁴ of B16F10 melanoma cells and 5x10⁴ cells breast adenocarcinoma. The treatment was performed with the collagen in different concentrations, administered by intraperitoneal during 40 days. Animals bearing the B16F10 melanoma and adenocarcinoma of Ehrlich treated with Collagen showed significant reduction of tumor mass and area respectively 79% and 68%. The survival rate calculated by the Kaplan-Meier test showed that treatment with the samples of collagen induced a significant reduction in mortality rate and reduction in number internal metastases. **Conclusion:** Collagen significantly reduced tumor growth, active caspase-3 and formation of metastases in

experimental models of melanoma and breast adenocarcinoma. Collagen Type I and products hydrolysates peptides with small molecular mass is a promising new weapon for cancer treatment. Financial Support: CNPq

Keywords: *Collagen Tipe I, Melanoma, Breast Cancer, Caspase-3, Tumor*

09.21- Characterization of Three Novel Splice Variants of the Reck Tumor and Metastasis Suppressor Gene: Correlation with Glioma Progression

Marina Trombetta Lima¹, Sheila Maria Brochado Winnischofer², Ana Claudia Oliveira Carreira¹, Marcos Angelo Almeida Demasi¹, Christian Colin¹, Mari Cleide Sogayar¹

1. IQ-USP, Instituto de Química - Universidade de São Paulo, Av. Prof. Lineu Prestes, 748 - Butantã - São Paulo - SP- Brasil -CEP: 05508-000. 2. UFPR, Universidade Federal do Paraná, Av. Cel. Francisco H. dos Santos, 210 CEP 81531-970

*RE*version-inducing Cysteine-rich protein with Kazal motifs (*RECK*) encodes a membrane-anchored protein that suppresses both invasion and metastasis by negatively regulating at least three MMP's, namely: MMP-9, MMP-2 and MT1-MMP. Matrix metalloproteinase (MMP) family members are directly involved in tumor invasiveness and metastasis. In general, relative levels of MMPs increase with tumor progression, with a positive correlation being observed between the abundance of *RECK* expression in tumor samples and better prognosis for patients with several types of cancers. Recent studies showed mutations in splicing *cis* regulatory elements and alterations in the cellular splicing regulatory machinery, leading to changes in the splicing pattern of several cancer related genes, such as *CD44*, *BRCA1*, *APC*, *p53* and *FHT*. In the present study, three novel alternative isoforms of the *RECK* tumor suppressor gene, namely *RECK B* (1,548bp), *RECK D* (1,737b) and *RECK I* (1,101bp) were isolated by RT-PCR and their expression profiles were investigated using quantitative real time RT-PCR assays in a normal tissue RNA panel and, also, during glioma progression. Our results show that *RECK* isoforms display independent expression patterns when compared to the canonical form, indicating a more complex role of both the canonical and alternative *RECK* isoforms in glioma progression. Support: FAPESP, CNPq, FINEP

Keywords: *Alternative-splicing, RECK, Tumor-supressor-gene*

09.22- Cadmium (Cd++) Inhibits the Gelatinolytic Activities Of MMP-2 and -9: A Possible Role in Cadmium-Induced Prostate Carcinogenesis

Livia Maria Lacorte¹, Carolina Sarobo¹, Elaine Manoela Porto², Jaqueline Carvalho Rinaldi¹, Luis Antonio Jr Justulin², Larissa Mayumi Ribeiro¹, Sérgio Luis Felisbino^{1,2}

1. UNESP, Sao Paulo State University, Department of Morphology, IB, Botucatu-SP. 2. UNICAMP, State University of Campinas, Department of Cell Biology, IB, Campinas-SP

Matrix Metalloproteinases are a set of neutral endopeptidases calcium and zinc-dependent. This family of enzymes plays a major role in the extracellular matrix components degradation and turnover in the most normal physiologic conditions and also in many diseases, such cancer. In this work, we evaluated the effect of cadmium, a divalent metal with known carcinogenic potential on prostate gland, on the MMP-2 and MMP-9 gelatinolytic activity. For such, MMP-2 and MMP-9 extracted from normal rat ventral prostate were processed for gelatin zymography. The cadmium effect was tested during incubations step at different concentration (5mM, 2mM, 50uM and 1uM of Cadmium chloride) with or without 5mM of calcium chloride. The gels were incubated for 20 hours at 37°C in a solution of 50mM pH 8.4 Tris-HCl containing the different concentration of cadmium and calcium described above. The presence of cadmium chloride in the incubation buffer inhibited the gelatinolytic activity of both MMP-2 and MMP-9 even in the calcium chloride presence. This result suggests that cadmium, such as magnesium, manganese and cobalt, has an inhibitory effect on MMP-2 and MMP-9 activity, in vitro. Although these metals not are essential for normal physiology, such as calcium and zinc, the prostate gland is the main organ where cadmium accumulates. Thus, the inhibitory action of cadmium on MMPs activity may, at long term, participates in the process of prostate carcinogenesis induced by cadmium. Further studies will address if prostates from cadmium-treated rats also present changes in MMP-2 and MMP-9 expression and gelatinolytic activity.

Keywords: *cadmium, prostate, cancer, metalloproteinases*

09.23- Treatment with Phosphoetanolamine Synthetic Induced Apoptosis Arrest in Cell Cycle Phases and Collagen Matrix Remodeling in Melanoma

Adilson Kleber Ferreira¹, Vanessa Martins¹, Ricardo Alexandre Azevedo¹, Fabio Marques², Adriano Radin², Gilberto Orivaldo Chierice³, Durvanei Augusto Maria¹

1. IBUT, Biochemistry and Biophysics Lab., Institute Butantan, AV. Vital Brazil 1500. 2. INRAD, Nuclear Medicine Center, Faculty of Medicine, USP, AV. Dr. Arnaldo. 3. USP, Technology of Polymers Lab., Analytical Chemistry, USP, AV. Trabalhador São Carlense

Introduction: Phosphoetanolamine is involved in the metabolism of phospholipids and responsible for maintaining the potential Deltapsi-m mitochondrial acting cell death signaling pathways. **Objective:** Evaluated effects antitumor Phosphoetanolamine Synthetic (PHO-S) in model B16F10 melanoma. **Materials and Methods:** The cytotoxic activity of PHO-S was tested in vitro in murine and human tumor lines (Skmel-28, Mel-85; Mewo and B16F10) by MTT colorimetric method. The model of implantation of the tumor cells were used 40 mice of strain C57BL-6J receiving 5x10⁴ cells B16F10 after 4 days treatment with (0.0117 and 0.0468 uM) from PHO-S (ip). The tumor volume was evaluated by scintigraphic with radioactive complex [[^{99m}Tc] (V) (DMSA) 2]). Part of lesions of tumor and metastasis internal were used for histological analysis (HE) of irrigation and endothelial vessels (Verhoeff), synthesis and content of collagen (Picrosirius), and determine the phases of the cell cycle (apoptosis, G0/G1, S and G2/M), by flow cytometry. **Results:** Showed selective cytotoxicity with IC₅₀% inhibitory concentration for cells of melanoma Skme-28 0.20mg/mL; 1.82mg/mL of Mel-85; Mewo of 2.39mg/mL and the murine melanoma B16F10

of 1.44mg/ml. Treatment of animals bearing a dorsal melanoma of PHO-S induced significant ($p= 0.0001$) reduction in tumor burden ($2.9 \pm 1.4 \text{ mm}^3$), compared to the control group ($13.8 \pm 6.6 \text{ mm}^3$), inhibiting the number of internal metastases. The cell cycle, induces PHO-S increase the proportion of cells in apoptosis in the concentration of 0.0117uM ($17.5\% \pm 2.3$), at a dose of 0.0468 uM decreased the proportion of quiescent cells (G0/G1) ($12.7\% \pm 4.06$). The ability of synthesis (S phase and G2/M) decreased significantly (P Conclusion: The PHO-S reduced volume of dorsal tumors, inhibition of tumor spread, and inhibition of proliferative and increased production of fibrillar matrix. During the cell cycle by flow cytometry of the treated tumors induced PHO-S "arrest" in the G0/G1 phase, non-proliferating and increasing the population of cells killed by apoptosis. Financial Support: FAPESP

Keywords: *Phosphoethanolamine, Apoptosis, Cell Cycle, Melanoma, Matrix*

09.24- Structural Analysis of the N-Terminal Fragment of Endostatin by Molecular Dynamics

Pedro H. M. Torres, Gabriel Limaverde Soares Costa Sousa, Pedro G. Pascutti

1. IBCCF-UFRJ, Instituto de Biofísica Carlos Chagas Filho - UFRJ, CCS, Cidade Universitária - UFRJ, Rio de Janeiro/RJ - Brazil

Endostatin is a potent antiangiogenic factor whose mechanism of action is not fully characterized. It was suggested that the activity of murine endostatin was retained on its 27 amino acids N-terminal fragment. This peptide coordinates a zinc ion by three histidine residues. When this metal is removed from the peptide, the antiangiogenic activity is abolished. In this work, we studied by Molecular Dynamics the N-terminal fragments of human and murine endostatin (N27hES and N27mES, respectively) to characterize key elements, like the zinc ion, that may have significant structural role on their biological function. Models built with PyMol, based on the crystallographic structures (1BNL and 1DY1), were simulated with and without zinc ion using GROMACS. An energy minimization protocol and three steps of molecular dynamics were used: (i) 500ps with positions restraint for peptide, (ii) 5000ps with all atoms free and (iii) 120ns for data gathering. Two additional N-terminal peptides of human endostatin were simulated: N25hES, without the last two residues, and N27hES/R4Q, with the mutation R4Q. We show that N27mES, when bound to the zinc ion, assumed well-defined secondary and tertiary structures in approximately 80ns, while the peptide without zinc collapsed in a compact fold without secondary structures. The conformation assumed by the N27mES has similarity with the functional β -hairpin of canstatin, a known anti-angiogenic protein derived from collagen IV. N27hES did not achieve a stable structure in the same amount of time in both simulations, with or without zinc. We postulated that this behavior was due to internal electrostatic repulsion between arginines 4 and 27. Confirming our hypothesis, simulations of N27hES/R4Q and N25hES presented stable β -hairpin conformations. Our study may help the development of new endostatin-based anti-angiogenic drugs.

Keywords: *Anti-angiogenesis, Endostatin, Cancer, Molecular Dynamics, Rational Drug-design*

09.25- Increased Expression of Hyaluronan Synthases by the Tumoral Microenvironment is Associated to Low Grade Non-Small Cell Lung Carcinomas

Vanessa Karen de Sá¹, Edwin Roger Parra-Cuentas¹, Eloisa Ribeiro Olivieri², Dirce Maria Carraro², Vera Luiza Capelozzi¹

1. FMUSP, Faculdade de Medicina da Universidade de São Paulo, Av: Doutor Arnaldo, 455 sala 1143 São Paulo-SP. 2. HCancer, Hospital do Câncer AC. Camargo, . Professor Antônio Prudente, 211 São Paulo - SP

Introduction/Objective: Hyaluronan, a tumor promoting extracellular matrix polysaccharide, is elevated in tumors including lung cancer, and associates with an unfavorable prognosis. The heterogeneity of HAS expression has been identified in tumors and could be related to the histological types and degree of differentiation. To explore possible contributors to the accumulation of hyaluronan, we examined the immunexpression of hyaluronan synthases (HAS1, HAS2 and HAS3) in frozen tissue obtained from non-small cell lung carcinomas (NSCLC). **Material and Methods:** We examined HAS1, HAS2 and HAS3 in tumor tissues from 12 patients with surgically excised NSCLC. We used frozen section. immunohistochemistry and histomorphometry to evaluate the amount of tumor areas staining for HAS1. HAS2 and HAS3. Tumor tissue from 3 patients submitted to surgical resection by non tumoral causes was used as control. Impact of these markers was tested on histological types and degree of tumor differentiation. **Results/Conclusion:** A significant direct association was found between HAS1 and HAS2 expression ($p=0.04$). HAS1 and HAS2 expression was significantly increased in adenocarcinoma compared to control and other histological types ($p=0.02$). HAS2 expression was lower in neuroendocrine carcinomas than in control and non neuroendocrine tumors, but this difference didn't achieve statistical significance ($p=0.42$). HAS expression was tested against histological degree, and an association was found between low grade tumors (well and moderate differentiation) with HAS2. We concluded that tumor staining for HAS2 may be related to prognosis of patients with NSCLC. **Financial Support:** FAPESP.

Keywords: *hyaluronan, lung cancer, Glycosaminoglycans, Hyaluronidase*

09.26- Inhibition of Cell Migration and Endothelial Cell Adhesion and Tubulogenesis by DM43, a Glycoprotein from *Didelphis Aurita* Serum

Marcela Ferreira Marques¹, Viviane Walleistein Mignoni¹, Camila Castro Figueiredo¹, Jonas Enrique Aguilar Perales², Patrícia Barbosa Jurgilas², Verônica Morandi¹

1. UERJ, Universidade Estadual do Rio de Janeiro, Departamento de Biologia Celular, IBRAG. 2. Fiocruz, Fundação Oswaldo Cruz,

Departamento de Fisiologia e Farmacodinâmica, IOC

Several animals, including the opossum *Didelphis aurita*, are resistant to snake venoms due to the presence of neutralizing factors in their blood. An antihemorrhagic protein named DM43, isolated from *D. aurita* serum, inhibits snake venom metalloproteinases through the formation of a noncovalent complex with these enzymes. DM43 is also a potential inhibitor of matrix metalloproteinase (MMPs). The MMPs are a family of extracellular endopeptidases that selectively degrade components of the extracellular matrix, and have been implicated in many physiologic and pathologic processes. Angiogenesis is an invasive process that requires proteolysis of the extracellular matrix, migration and proliferation of endothelial cells. The angiogenic response is critical for progression of wound healing, rheumatoid arthritis and is also a prerequisite for tumor growth and metastasis formation. In this study, we investigated the effect of DM43 in cell adhesion, proliferation, migration, invasion and differentiation. Using a prostate cancer cell line (DU-145), two breast cancer cell lines (MCF-7 and MDA-MB-231), and human umbilical vein endothelial cells (HUVEC), we first tested whether DM43 could be a potential inhibitor of cell migration. We observed that DM43 (4000 ng/ml) inhibits chemotaxis of all cancer cell lines (MDA-MB-231- 45% ,MCF-7 -58% and DU-145 - 70%), as well as in human umbilical cells (HUVEC- 66%). DM43 also significantly decreased the adhesiveness of HUVEC cells to gelatin in a dose –dependent manner (250-4000ng/ml), with the maximal effect at 4000ng/ml (29 % of inhibition), as demonstrated by the MTT method. Finally, DM43 also inhibited significantly the formation of capillary-like structures by HUVEC, on a 3D-Matrigel matrix. This study suggests that DM43 has a potential as a therapeutic agent in pathological situations where angiogenesis is stimulated, such as tumor growth and progression. Supported by: CNPq, FAPERJ, CAPES.

Keywords: *Angiogenesis, Endothelial cells, Extracellular matrix, Matrix metalloproteinase, Tumor cells*

09.27- Screening of Flavonoids Effects of Growth and Aggregation of Human Glioblastoma Initiating Stem Cells: Inhibitory Effect of Quercetin on Cellular Adhesion and Migration

Bruno Penas Pitanga, Balbino Lino Santos, Silvia Lima Costa

1. UFBA (LabNq), Universidade Federal da Bahia, Lab Neuroquímica e Biologia Celular, ICS/UFBA, Av Reitor M Calmon, Salvador, Brazil

Gliomas are the most frequent and malignant among human intracranial tumors. Despite radical surgery, the prognosis with radiation therapy and conventional chemotherapy remains low due to invasion of surrounding brain tissues. The isolation from human biopsies of malignant cells that express markers of neural stem cells support the existence of tumour stem cells within gliomas. As our previous study have benn suggested plant derived flavonoids as inhibitors of growth of human and murine glioblastoma cell lines, in this study we investigated the effect of fifteen commercially disponibile flavonoids on vitality and adhesion on a human cell line of tumour initiating cells (TG1) and in viability and invasion of the human glioblastoma cell line U251. As revealed by WST and MTT test after 72 h exposure to

1-100 μ M flavonoids. Depending on the degree of methylation and hydroxylation flavonoids inhibited dose-dependently the growth and viability of both glioblastoma cell lines, and between them one of the most active was flavonoid quercetin. Then we tested by time lapse microscopy the effect of quercetin on adhesion of spheroids of TGI cells and by phase microscopy the migration of synchronized U251 cells on different ECM support (laminin, collagen, collagen plus fibronectin) through the use of a standard in vitro monolayer wound assay. Compared with cells in control conditions (vehicle DMSO 0.1%) 50 μ M quercetin reduced the aggregation of TGI cells spheroids since 15h after exposure. Moreover 50 μ M quercetin also reduced significantly the closure of the wounded area indicating inhibition on U251 glioblastoma cells migration. These results suggest flavonoids quercetin as inhibitor of growth of glioblastoma tumor initiation cells and may find an application to treatments for chemotherapy resistant malignant gliomas. Supported by CNPq, CAPES and INSERM U-894.

Keywords: *Glioblastoma, Flavonoid, ECM, adhesion, migration*

09.28- Polyhydroxylated Flavonoids Regulate Mmps Activity, MEC Expression, and Invasion of Human Glioblastoma Cells

Balbino Lino Santos, Mona das Neves Oliveira, Milton Albuquerque Franco Souza, Paulo Lucas Cerqueira Coelho, Bruno Penas Seara Pitanga, Maria de Fátima Dias Costa, Ramon dos Santos El-Bachá, Sílvia Lima Costa

1. UFBA (LabNq), Universidade Federal da Bahia, Lab Neuroquímica e Biologia Celular, ICS/UFBA, Av Reitor M Calmon, Salvador, Brazil

Malignant gliomas are the most common primary brain tumors in adults and its incidence has increased dramatically over the past several decades. Due to its invasive character, and despite advances in standard therapy, surgery is unable to remove all tumor tissue, and the prognosis for patients with gliomas remains poor. Matrix metalloproteinases (MMPs) have been implicated as important factors in the control of the invasive capability of gliomas. Flavonoids are polyphenolic compounds present in a large variety of plants, and have been presented antitumoral activities. In this study we investigated the effect of polyhydroxylated flavonoids chrisyn (5,7- dihydroxiflavone), 3',4'-dihydroxiflavone, apigenin (4',5,7-tetrahydroxiflavone), and kaempferol (3,4',5,7-tetrahydroxiflavone), on MMPs activity, ECM component fibronectin expression, invasion, and viability of GL-15 human glioblastoma cells. Cells were cultured in serum free supplemented DMEM and after 24 h exposure to 50 μ M flavonoids were analyzed by Immunocytochemistry for fibronectin expression and by zymography to determine MMPs activity. Cell viability was determined by MTT test. Synchronized GL-15 cells were cultured on different ECM supports (laminin, collagen, collagen plus fibronectin), treated with flavonoids (50 μ M) and migration of glioblastoma cells was investigated through the use of a standard in vitro monolayer wound assay, and closure of the wounded cell free area was followed after 1-5 days. Compared with cells in control conditions (vehicle DMSO 0.1%) flavonoids reduced viability of GL-15 cells and induced changes on pattern of extracellular fibronectin expression. Phase contrast microscopy and Rosenfeld staining showed that the cell-free wound gap of GL-15 control monolayer

healed faster, and more cells appeared in the wounded gap on collagen plus fibronectin support, which represented enhanced migratory activity towards the wounded area. However, in the flavonoids treated groups, the closure of the wounded area was significantly slower indicating inhibition on glioblastoma cells migration. Moreover, pro-MMP2 and MMP-2 activity were efficiently inhibited in the presence of polyhydroxylated flavonoids. Taken together these results suggest Polyhydroxylated flavonoids as inhibitor of glioblastoma cells growth and invasion and may find application to traditional chemotherapeutic treatments for malignant gliomas. Supported by CNPq, CAPES and INSERM U894.

Key words: *glioblastoma, flavonoids, MMPs, fibronectina, invasion*

09.29- Laminin Peptide SIKVAV Regulates Migration, Invasion and Protease Activity of Adenoid Cystic Carcinoma Cells Through Integrins, Fak and ERK

Letícia Nogueira de Gama Souza, Milza Oliveira de Assis, Vanessa Moraes Freitas, Ruy Gastaldoni Jaeger

1. ICB-USP, Instituto de Ciências Biomédicas/Universidade de São Paulo, Av. Prof. Lineu Prestes 1524

Adenoid cystic carcinoma is a malignant salivary gland which presents prominent expression of extracellular matrix molecules. The extracellular matrix and basement membrane play an important role regulating phenotypic characteristics of neoplasms. Laminin is a glycoprotein predominantly expressed in basement membrane, which influences tumor behaviour. This molecule also contains cryptic sites with biological activity, which can be available for cells through proteolytic cleavage. During invasion, neoplastic cells produce proteolytic enzymes, as matrix metalloproteinases (MMPs) that degrade laminin and other extracellular matrix components. This process promotes release of fragments and bioactive peptides of laminin that can influence tumor biology. One of these peptides is SIKVAV (carboxy terminal domain of the laminin- α 1 chain) which is involved in malignant transformation, cell proliferation, angiogenesis and protease activity in a number of cell types. In this study we investigated regulation of migration, invasion and protease activity by the peptide SIKVAV in a cell line (CAC2) derived from adenoid cystic carcinoma. Monolayer wound assays and Transwell migration assays showed that SIKVAV increased migration of CAC2 cells compared to controls. This peptide also stimulated invasion in Boyden chambers coated with Matrigel. Invasion depends on protease activity. SIKVAV increased secretion of MMPs 2 and 9 compared to controls. We also studied putative receptors involved in SIKVAV activities. siRNA knockdown of α 3 and β 1 integrins inhibited SIKVAV-induced migration and protease activity. Different signaling pathways could be related to SIKVAV effects in CAC2 cells. This peptide increased both phospho-ERK and phospho-FAK compared to controls. We propose that SIKVAV increases migration, invasion and protease activity of CAC2 cells through integrin heterodimer α 3 β 1. Signal generated by SIKVAV is transduced by FAK and ERK1/2 signaling pathways. Supported: FAPESP(2006/57079-4, 2008/57103-8 and 2009/06299-2) and CNPq (304868/2006-0 and 470622/2007-5).

Key words: *adenoid cystic carcinoma, extracellular matrix, invasion, laminin, matrix metalloproteinases*

09.30- BRAF- Mutation and Expression of Hyaluronan and Hyaluronidase in Differentiated Thyroid Carcinoma

Valquíria Pereira Medeiros¹, Juliana Augusta Albieri Dominato¹, Lyvia Ferreira Ebner¹, Daniela Bortoletto Martinatti¹, Aline Mendes¹, Elsa Yoko Kobayashi¹, Edna Teruko Kimura², Helena Bonciani Nader¹, João Roberto Maciel Martins¹

1. UNIFESP, Universidade Federal de São Paulo, Rua Três de Maio, 100 - 4o andar - Vila Clementino – SP. 2. USP, ICB-Universidade de São Paulo, Av. Prof. Lineu Prestes, 1524 - 4o andar - sala 414 - Pinheiros - SP

Differentiated thyroid cancers (papillary - PTC and follicular - FTC) are the most common endocrine malignancies. In PTC, about 70% of cases has a known genetic event, specially RET/PTC rearrangement and BRAF mutation. Point mutations of BRAF are found in about 40-50% of PTC and have been implicated with poorer outcome. The development and progression of human tumors is accompanied by various cellular, biochemical and genetic alterations which include tumor cells interaction with extracellular matrix molecules including hyaluronan (HA). HA is a high molecular weight linear glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and N-acetyl-Dglucosamine. Its implication in malignant transformation, tumor progression and with the degree of differentiation in various invasive tumors has been reported and well accepted. There are evidences that tumors cells from different origins can degraded HA by specific enzymes, Hyaluronidases (Hyase), generating low molecular weight fragments. These fragments have distinct functions in tumor biology, including angiogenesis, which have been implicated in tumor invasion and progression. In this study, we investigate if there is differential expression of HA and Hyase in two PTC cell lines, one positive for BRAF mutation (NPA) and the other with RET/PTC rearrangement (TPC-1). Cell lines were cultured in F12 medium supplemented with 10% bovine fetal serum until 90% confluence. Afterwards, culture medium and cellular extract were analyzed separately. HA was analyzed by an ELISA-like fluoroassay using a specific biotinilated HA-binding protein as probe. Hyase activities from culture media were determined by an ELISA-like fluoroassay and compared with standard testis hyaluronidase in pH 4.0 and 7.0. TPC-1 synthesized significantly more HA if compared to NPA (213.2 ± 9.4 versus 10.3 ± 1.1 ng/mg protein). Interestingly, a distinct profile of Hyase activities was observed when the two cell lines were compared. TPC-1 showed an increased enzymatic activity in pH 7.0 when compared to NPA (14.3 versus 1.2 mUI/mL) while in pH 4.0 the enzymatic activity was higher in NPA (25.6 versus 1.1 mUI/mL). Although preliminary, our results provide evidence for a process that represents a probable activation mechanism, related to BRAF mutation, which leads to the promotion and maintenance of the higher malignant phenotype of thyroid carcinoma bearing this mutation. Supported by FAPESP and CNPq

Keywords: *thyroid cancer, BRAF mutation, hyaluronan, hyaluronidase, glycosaminoglycan*

10. ECM AND CELL THERAPY

10.1- Effect of Dermatan Sulfate in the Migration and Proliferation of Endothelial Progenitor Cells after Arterial Injury in Mice

*Juliana Aparecida Preto de Godoy¹, Daniel Bueno Block¹, Douglas M Tollefsen²,
Cristina Pontes Vicente¹*

1. UNICAMP, Universidade Estadual de Campinas, Rua Monteiro Lobato, 255 - Campinas - SP - Brasil - CEP 13083-862. 2. WUSM, Washington University Medical School, Division of Hematology, Campus Box 8125, 660 South Euclid Avenue

Coronary artery disease is the leading cause of mortality in the western world. Some of the principal treatments for this disease include angioplasty, stenting and arterial bypass. The success of these interventions remains limited by negative remodeling resulted in lumen restenosis; approximately 50% of the patients presents restenosis after 6 months of the vascular injury. Neointima formation is characterized by endothelial dysfunction that is mainly associated with elevated smooth muscle cells (SMC) proliferation. Endothelial progenitor cells are able to migrate to the site of endothelial lesions and differentiate into mature endothelial cells helping the recovery of the injured endothelium; they are characterized by markers like CD34 and CD133. Vascular repair by circulating progenitor cells of ongoing vascular injury may be essential for vascular integrity and function. Bone marrow is the main source of these cells. It has been described that transfusion of bone marrow cells (BMC) to mice after arterial injury can help restore the endothelium, avoiding the neointima formation and restenosis. The mechanisms of migration and differentiation of these cells to the site of injury are not well established. We studied the relationship between the administration of dermatan sulfate (DS) and injection of bone marrow cells (BMC) in neointima formation. For this we injured the left common carotid of C57BL/06 mice using a wire guided probe that mimics angioplasty. We studied 4 groups, control (1), DS injected (2), BMC injected (3) and BMC + DS injected (4). We analyzed the neointimal area, cell proliferation in media and intima, and migration of inflammatory cells in different days after lesion. We observed that the number of cells in medial and intimal area in mice that received only the BMC were higher than that in the other groups in all analyzed times. The percentage of neointima formation was 100%; in group 1, 34,45% in group 2; 46,02% in 3, and 26,63% in 4. The BMC injected contained 0,5 % of cells CD34/CD133 positive, what is according to many authors to identify the progenitor cells presents in the BM. We can conclude that the DS inhibits the initial inflammatory process after arterial injury and, when administrated together with BMC, it decreases neointimal area avoiding SMC proliferation. These results suggest that DS can help in the migration and proliferation of the BMC, by inhibiting macrophages migration and SMC proliferation in the site of injury.

Key words: bone marrow cells, angioplasty, neointima, smooth muscle cells, CD34

10.2- Cartilage Progenitor Cells and Alginate: A Tissue Engineering Approach

Ronaldo José Farias Corrêa do Amaral¹, Carolina Silva Gouveia Pedrosa², Karina Ribeiro da Silva², Marcelo Aniceto³, Cesar Claudio-da-Silva³, Leandra Santos Baptista², Radovan Borojevic²

1. ICB - UFRJ, Instituto de Ciências Biomédicas - UFRJ, ZIP CODE: 21941-902. 2. DIPRO - INMETRO, DIPRO - INMETRO, Rio de Janeiro, RJ, Brasil. 3. HUCFF - UFRJ, Departamento de Clínica Médica - HUCFF - UFRJ, Rio de Janeiro, RJ, Brasil

Cartilage is a tissue with low regeneration capacity. After a wound, it is generally substituted by a non-functional fibrous tissue, the fibrocartilage. Chondrocytes may lose their chondrogenic phenotype *in vitro*, so that progenitors cells have been investigated as alternative sources of cells for cartilage engineering. The universal strategy of tissue engineering is to seed cells into three-dimensional scaffolds. The aim of this study is to isolate and characterize human cartilage progenitor cells and to associate them with scaffolds that could be used in cartilage bioengineering protocols. Cartilage samples were obtained from human nasal biopsies during aesthetic surgery procedures. Cells were isolated after collagenase digestion (2mg/ml for 2 hours), expanded *in vitro* at 37°C with 5% humidified CO₂ and characterized by morphological analysis, expression of mesenchymal stem cells surface markers, proliferation and differentiation assays. Finally, they were encapsulated in 2% alginate beads, and incubated in 0,1M CaCl₂ in order to form a hidrogel. Cartilage progenitor cells were negative for CD34 and CD45 and positive for CD44, CD73 and CD105. They showed osteogenic, chondrogenic and high proliferation capacity. Notably, they also revealed a spontaneous chondrogenic differentiation, different from other progenitor cells described in the literature. When encapsulated in alginate hidrogels, they acquired a rounded morphology, typical of chondrogenic lineage cells *in vivo*. This work showed that cells isolated from human hyaline cartilage have a high chondrogenic capacity and could be successfully associated with alginate. In parallel, our laboratory has developed a fiber mesh scaffold based on silk fibroin with interesting physical properties and already associated it with alginate. In the future, cartilage progenitor cells previously encapsulated in alginate will be associated to our fiber mesh in order to generate a cartilagenous tissue *in vitro*.

Key words: *alginate, cartilage, progenitor cells, tissue engineering*

10.3- Mononuclear Bone Marrow Cell Transplantation Decreases Collagen Accumulation in The Liver of Fibrotic Rats

Simone Nunes Carvalho, Genilza Pereira Oliveira, Alessandra Alves Thole, Camila Alarcão, Ana Carolina Stumbo, Carlos Eduardo Caetano, Ruy Garcia Marques, Laís Carvalho

1. UERJ, Universidade do Estado do Rio de Janeiro, Av Professor Manoel de Abreu, 444 3° andar - Maracanã - RJ

Hepatic fibrosis is a pathogenic process that results from a chronic lesion to the hepatic parenchyma, generally due to intoxication, congenital biliary atresia or hepatitis. The main feature of hepatic fibrosis is the process of collagen accumulation either in the hepatic parenchyma and/or portal areas, with massive hepatocyte death and substitution by a fibrous extracellular matrix. Chronic fibrosis often leads to loss of hepatic function and development of cirrhosis and hepatic failure. Liver transplantation is the most effective treatment to hepatic failure, but it is limited by organ shortage. Recently, transplantation of bone marrow stem cells has emerged as an alternative to treat subjects with moderate hepatic fibrosis and may help to slow the progression of the disease and improve hepatic parameters [1]. In this study, bone marrow mononuclear (BMMN) cells were injected in rats with hepatic fibrosis induced by bile duct ligation (BDL), and collagen levels were analyzed. Rat bone marrow cells from tibias and femurs were purified using a Ficoll-gradient-centrifugation, labeled with Cell Tracker and injected in the jugular vein of fibrotic rats obtained 14 days after BDL. After 7 days, rats were sacrificed, livers were collected and then paraffin sections were stained with Picro Sirius. To quantify liver fibrosis, 15 random fields per animal were captured in a light microscope with a CCD camera and then analyzed with the software Image Pro. The percents of fibrotic areas were then compared with the control groups (normal animals, 14-day and 21-day liver fibrosis) using ANOVA and Kruskal-Wallis post-test. In this study, liver fibrosis resulted in abnormal collagen accumulation in portal areas and in the formation of collagen-rich septa between hepatic lobules. These features were evident 14 days after BDL, and continued to progress in the 21st day. However, fibrotic rats treated with BMMN cells had fewer septa in the parenchyma, displaying less collagen ($3,31\% \pm 1,85$) than their controls (rats with 21-day fibrosis, $5,3\% \pm 1,05$, and rats with 14-day fibrosis, $4,7\% \pm 1,27$). Therefore, BMMN cells transplantation decreased collagen accumulation in fibrotic rats after BDL. We hypothesize that BMMN cells may help restore the normal balance of the extracellular matrix components in these livers, decreasing collagen accumulation. Acknowledgements: CNPq, CAPES and FAPERJ. Reference: [1] Levicar, N. et al, 2008. Cell Proliferation 41, 115-125.

Keywords: *mononuclear bone marrow cells, liver fibrosis, bone marrow stem cells, collagen*

10.4- Reevaluting the Number of Colony-Forming Units-Fibroblast in the Adult Human Bone Marrow

Eric Spinetti¹, Veronica Vianna¹, Barbara Cristine Ribeiro¹, Regina Mendes¹, Maria Isabel Doria Rossi², Marco Bernardo Cury Fernandes¹, Maria Eugenia Leite Duarte¹, Wallace de Mello¹, Alex Balduino¹

1. INTO, Instituto Nacional de Traumatologia e Ortopedia, Rua Washington Luis, 61/6º andar. 2. UFRJ, Universidade Federal do Rio de Janeiro, Rua Professor Rodolfo Paulo Rocco

In the bone marrow, stromal cells play an important role in microenvironment formation and hematopoietic stem cells self-renewal, proliferation, and differentiation. The frequency of these progenitors in healthy patient's bone marrow aspirates is 30 ± 12 per 106 cells. CFU-Fs can also be found in umbilical cord blood and peripheral blood, and adipose tissue in much lower and higher frequencies, respectively. In the present study we isolated the mesenchymal population from the acetabular reaming bone marrow fragments. Acetabular reaming were

obtained from patients with ages range between 20 and 60 years, mal and female, with no osteometabolic diseases, undergoing total hip arthroplasty. Fragments were suspended and homogenized in saline solution (5 times), supernatant was recovered and centrifuged. Pellet was resuspended, cells were counted, and 200,000 cells were plated per well. Three days later, supernatant was discarded and the adherent population was incubated in fresh medium for extra 10 days. Colonies were fixed, stained, and counted. Colony forming efficiency (CFE) was presented as colonies per million of nucleated cells. Phenotypic analyses were performed by a hemogram, RT-PCR and FACS analyses. All cells isolated expressed higher levels of CD105, CD44, and CD13. Less than 5% of cells expressed both CD45 and CD34. RT-PCR analyses indicated that mesenchymal population is heterogeneous, expressing osteogenic, adipogenic, and chondrogenic markers. The average of CFE was 233 (30 – 720), almost seven fold higher than that regularly described in the literature. Our data shows that cells isolated from the acetabular reaming (surgical waste) present similar phenotype to that described in the literature, but indicates that skeletal progenitors frequency is much higher than usually believed. CFE values may provide useful insights into reevaluating the way mesenchymal cells are obtained.

Key words: *mesenchymal cells, skeletal progenitors, Bone marrow*

10.5- Effect of Leucurogin, A Recombinant Disintegrin, Upon Fibroblast Adhesion

Debora Ayame Higuchi^{1,3}, Meire Coelho de Almeida^{1,3}, Paola Zigrino², Cornelia Mauch², Jorge Luiz Pesquero⁴

1. UMC, University of Mogi das Cruzes, Av. Dr. Cândido Xavier de Almeida Souza, 200, Mogi das Cruzes, SP, Brazil. 2. CMMC, University of Cologne, Kepener Str. 62, Cologne, Germany. 3. Proteobras, Proteobras Desenvolvimento Tecnológico Ltda, Av. João XXIII, 1160, Cesar de Souza, Mogi das Cruzes, SP, Brazil. 4. UFMG, University of Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, Belo Horizonte, MG, Brazil

Disintegrins show specific sequences that can bind with integrins, inhibiting cellular processes like adhesion, migration and proliferation in normal and tumor cells. Leucurogin is one recombinant disintegrin produced from a salivary gland of *Bothrops leucurus* snake. This work was carried out to characterize the action of leucurogin upon murine and human adhesion in fibroblasts cell lines, and verify the competition of the recombinant protein with collagen type I in the adhesion process. To analyze the adhesion process were used human (hFb) and murine (3T3) fibroblasts. Cells were cultivated in plates containing 10% fetal calf serum (FCS) in humid atmosphere with 5% CO₂ at 37°C. After trypsinization and verification of cells viabilities, cells were added (40 x 10³ cells/well) in plates of 96 wells, previously incubated with collagen type I (40 µg/mL), leucurogin (10 µM) or 100% FCS. For negative control were used wells without previous incubation. The positive control for human fibroblast was used collagen type I and for murine, FCS. In the competition test, wells were covered with extracellular matrix components (ECM). Then, the cells, previously incubated with leucurogin at concentrations in the range 0.1-10 µM for 1h, 37°C were added to the wells. The unspecific interaction was blocked by incubating the wells with inactive 2% BSA

for 1h at 37°C. The cellular adhesion was analyzed by the absorbance in 595 nm after labeling the adhered cells with violet crystal (0.5%). Low level of hFb adhesion was observed mediated by leucurogin (OD 595 = 0.13), when compared with the collagen, positive control (OD 595 = 0.28). However for the 3T3, the result of adhesion mediated by leucurogin (OD 595 = 0.1) was similar to that observed to the control, FCS (OD 595 = 0.09). In the competition assay between leucurogin and collagen, recombinant disintegrin inhibited, in a dose-dependent manner, the adhesion of collagen with both fibroblasts cell lines. At 10 μ M leucurogin was able to reduce 50% adhesion in hFb and 90% in 3T3. The results indicate that indicate that leucurogin is capable to inhibit cellular processes dependent of collagen I-integrin interaction. Financial support: Fapesp, CNPq, Capes, Finep, Faep and DAAD.

Keywords: *disintegrin-like, integrins, collagen type I, cell adhesion*

10.6- Mesenchymal Stem Cells from Human Adipose Tissue Promotes Regeneration of SCI

Karla Menezes, Leandra Baptista, Karina Ribeiro, Carolina Pedrosa, Daiana Vieira, Marcos Assis Nascimento, Gabriel Gomes Maia, Raphael Siqueira Santos, Maria Isabel Rossi, Tatiana Lobo Coelho Sampaio

1. UFRJ, Universidade Federal do Rio de Janeiro, Av. Brigadeiro Trompowsky sem número, centro de ciência da saúde Ilha do fundão

Spinal cord injury causes profound changes in the nervous microenvironment that culminate in axonal degeneration and demyelization. In the acute phase, bleeding causes local hypoxia, which, combined with the production of inhibitory molecules and inflammatory mediators, lead to the destruction of neuronal tissue. Mesenchymal stem cells from adipose tissue (AT-MSC) have been shown to differentiate into different cell types and to participate in the angiogenic process. Here we investigate the effect of human AT-MSC in SCI. Acute local injection of hAT-MSC was performed after thoracic compression or transection (105 cells diluted in 10 μ l of DMEM). In both models there was a significant improvement in locomotor function, and the final BBB score increased from 17.75 ± 0.75 to 19.75 ± 0.25 in compression and from 4.12 ± 0.23 to 9.6 ± 1.14 in complete transection. Morphological analyses of the experimental animals showed that treatment reduced the area of the cystic cavity and increased the number of cell bodies and fibers labeled with the neuronal regeneration marker GAP-43. It was also verified an increase in the amount of small blood vessels along the central canal, as well as in white and gray matters. Neovascularization of the spinal parenchyma could explain the beneficial effects of hAT-MSC, since it would increase the oxygen supply to the cells, thus preventing the progression of ischemic necrosis. Finally, we observed no sign of rejection of grafted human cells, which confirms the immunomodulatory character previously proposed for MSCs.

Keywords: *Mesenchymal stem cells, spinal cord injury, axonal regeneration*

10.7- Characterization of Bone Marrow Stromal Cells as a Source for Tendon Graft Recellularization

Livia Mendonça¹, Eric Spinetti¹, Veronica Vianna¹, Barbara Ribeiro¹, Eduardo Sousa¹, Regina Silva¹, Marco Cury¹, Maria Eugênia Duarte^{1,2}, Alex Balduino¹, Wallace de Mello¹

1. INTO, Instituto Nacional de Traumatologia e Ortopedia, Rua Washington Luis, 61 CEP 20230-024 Rio de Janeiro. 2. UFRJ, Universidade Federal Do Rio de Janeiro, Av Brigadeiro Trompovski s/n Rio de Janeiro

It is well established that bone marrow stromal cells (BMSC) may differentiate into bone, cartilage, smooth muscle, adipose and tendon tissues, being an important tool for tissue bioengineering and regenerative surgery. Tendon grafts are currently used to replace injured tissue, but their application is limited by the morbidity, in patients using autografts, or immunologic response against allografts. Over the past few years, protocols using tendon allografts have risen as an alternative source for tendon and ligament replacement. These tissues are decellularized, removing almost all allogeneic antigens. Then cell injection into acellularized tendon scaffolds may maintain tissue homeostasis and biomechanical properties by expression of both the fibrillar and non-fibrillar matrix. In the present work, we intend to characterize BMSC cultures obtained from acetabular reaming fragments in order to improve tendon allografts. Bone marrow was washed in CMF solution and cell pellet was resuspended in IMEM SBF 10% during three days. After this step, supernatant was discarded and cells cultivated during more 10 days. Flow cytometry showed an intense staining for CD105 and CD146, two markers present on mesenchymal cells. RNA extraction was performed for osteogenic, chondrogenic, adipogenic, tenogenic, myogenic and stemness markers. Scx is a transcription factor that induces the expression a tenocyte proliferative protein, Tenomodulin, while Fibromodulin and Biglycan are proteoglycans present in tendon stem cell niche. These Cells express tenogenic markers scx, tenomodulin and Fibromodulin but not Biglycan. Moreover, they were positive for all lineages studied and the embryonic stem cell marker Oct-4. These data point to a potential BMSC population more efficient for tendon repair, since it is described on literature that BMSC differentiate in tenocytes, expressing tenomodulin, under stimulation with BMP-12. In the near future, we intend to optimize protocols for acellularization and recellularization for better designing therapeutical strategies, including understanding on BMSC behavior *in situ*.

Keywords: BMSC, scx, Tenomodulin, Tendon

10.8- Laminin Alpha5 Expression after Human Myoblast Transplantation Into RAG-/-Gammac-/- Mice

Ingo Riederer^{1,2}, Elisa Negroni², Marina Trajano¹, Ariany Oliveira Santos¹, James DiSanto³, Wilson Savino¹, Vincent Mouly², Suse Dayse Silva-Barbosa¹, Gillian Sandra Butler-Browne²

1. Fiocruz, Lab pesquisas sobre o timo/IOC/Fiocruz, av Brasil 4365 Manguinhos Rio de Janeiro, RJ - Brasil - Caixa-Postal: 906. 2. UPMC, UMRS 974 INSERM-Paris 6-CNRS UMR

7215 Institut de Myologie, Bâtiment Babinski 47, boulevard de l'Hôpital. 75651 Paris Cedex 13 France. 3. Pasteur, Institut Pasteur, 25-28, rue du Docteur Roux - 75724 PARIS CEDEX

15

Myoblast transplant therapy (MTT) can be envisioned as a clinical alternative in the treatment of several myopathies. One of the problems that remain to be solved to improve the efficiency of MTT is the massive early cell death, low proliferation and limited migration of transplanted cells. Cell contact with proteins of extracellular matrix (ECM) can protect cells from death, and increase cell proliferation and migration. Laminin (LN) is an ECM protein and the major component of muscle basal membrane. Different LN isoforms are involved during muscle development and regeneration. In this work, we decide to study LN expression during human myoblast differentiation. In vitro, the isoform LN-alpha5 is expressed only from 3 days after differentiation induction and LN-alpha2 is expressed later, from 7 days. Interestingly, LN-5 was strongly detected in fused myoblasts (myotubes) but weaker in not fused cells, linking this isoform with the differentiation program. However, when we used a polyclonal antibody to LN, a positive staining was always detected, in proliferation medium as well in differentiation medium, indicating the expression of other LN isoforms. We recently showed that the immunodeficient mice RAG-/-gammaC-/- is an attractive and efficient recipient to support engraftment of human myoblasts. In this model, 5x10⁵ human myoblasts were injected into the Tibialis Anterior (TA) muscles of RAG-/-gammaC-/- recipients. Mice were sacrificed after 0h, 1h, 3h, 6h, 24h, 12h, 3 days, 5 days and TA muscles were analyzed by immunofluorescence. After cell injection we observed a progressive LN deposition, with a polyclonal antibody, in the area of transplanted cells. During this kinetics, no LN-alpha2 was detected. On the other hand, using the antibody against LN-alpha5, we clearly detected this isoform from day 3, time point where we detected the differentiation of the human injected myoblasts. This data relate the expression of this isoform with the differentiation program and corroborate the in vitro results. We have recently demonstrated a precocious differentiation of the transplanted human myoblasts, phenomena which could impairs the efficacy of MTT. The relation of the LN-alpha5 isoform expression with the differentiation of human myoblasts emphasize the importance of ECM components in muscle biology and will certainly be important to design new strategies for cell mediated therapy.

Keywords: *Cell Therapy, Laminin, Myoblast differentiation, Myoblast Transplantation, Skeletal Muscle*

11. ECM AND HISTOLOGY AND HISTOPATHOLOGY

11.1- HMG-CoA Reductase Inhibitors Differently Affect Rat Cutaneous Wound Repair

Tatiana Pádua¹, Thaís Amadeu^{1,2}, Andréa Monte Alto Costa¹

1. UERJ, Universidade estadual do rio de janeiro, Rua Professor Manoel de Abreu 87 fds, Vila isabel, Rio de Janeiro. 2. FIOCRUZ, Fundação Oswaldo Cruz, Av. Brasil, 4365 - Manguinhos, Rio de Janeiro

Statins are HMG-CoA reductase inhibitors that have immunomodulatory activities and pleiotropic effects but their role on cutaneous wound healing is unclear. This study investigated the effects of three statins on cutaneous wound repair. Rats were separated into control (received water only), simvastatin, pravastatin and atorvastatin groups (20mg/kg/day). Statins were administrated by gavage 10 days preoperatively until the end of the experiment. Eleven days after the beginning of the treatment, an excisional wound was performed. The wound area was measured to evaluate wound contraction and re-epithelialization and animals were sacrificed 7 (n=20) or 14 (n=20) days after wounding. Hematoxylin-eosin, picrosirius red, immunohistochemistry to alpha-smooth muscle actin, CD-68, and 4-hydroxynonenal (marker of lipid peroxidation), hydroxyproline analysis and determination of NO₂- in serum were done. All statins (simvastatin, pravastatin and atorvastatin) accelerated wound contraction 7 days after wounding, but 14 days after wounding, the wound contraction was higher in pravastatin, and atorvastatin showed delayed wound contraction. The re-epithelialization was higher only in simvastatin and pravastatin groups. Seven days after wounding the number of CD68+ cells was higher in the pravastatin and simvastatin groups; the number of neutrophils was lower in the pravastatin and atorvastatin groups compared to the control group, and in pravastatin than simvastatin group. In the deep area of the granulation tissue, both simvastatin and pravastatin groups presented a higher density of thickened red collagen fibers compared to the other groups in 7 days after wounding. Fourteen days post wounding, hydroxyproline concentration was lower in the simvastatin and atorvastatin than control group. The volume density of myofibroblasts (Vv[myof]), 7 days after wounding was lower in the simvastatin than pravastatin group; 14 days post wounding, statins treatment reduced the Vv[myof], mainly in the pravastatin and atorvastatin; and the simvastatin increased the Vv of blood vessels. The statin treatment also reduced the number of 4-HNE positive cells, mainly in the simvastatin 7 days after wounding, and in the pravastatin and simvastatin 14 days post wounding. Data suggest that statins present different effects on rat cutaneous wound repair. Supported by CAPES and CNPq.

Keywords: *Rat, Skin, Statins, Wound Healing*

11.2- Healing of Ischemic Wounds in Young and Mature Rats

Janaina Georgii¹, Thaís Amadeu^{1,2}, Andréa Monte-Alto-Costa¹

1. UERJ, Universidade do Estado do Rio de Janeiro, Av. Professor Manoel de Abreu, 444, 3º andar, 20550-170, Rio de Janeiro, Brasil. 2. FIOCRUZ, Fundação Oswaldo Cruz, Av. Brasil, 4365 - Manguinhos, Rio de Janeiro 21040-360

Cutaneous wound repair is an interactive process, in which several complementary events occur. When one of these events is prolonged or delayed, the next stage of the repair is disturbed and the healing will be impaired, developing chronic wounds. Considering that wound repair in young and mature animals presents differences, the aim of this study was to compare the progression of impaired ischemic wound repair in young and mature rats. Young and mature male Wistar rats (3 and 7 months old, respectively) were separate in control and ischemic groups. For ischemia, two parallel incisions were done, and skin was separated from the underlying tissue; incisions were sutured, and an excisional wound was performed

between incisions. Wound contraction and re-epithelialization were measured. Wounds were collected 14 days after wounding, and tissue sections were stained with hematoxylin-eosin, sirius red or toluidine blue and immunohistochemistry to alpha-SMactin and PCNA was performed. Wound contraction and re-epithelialization did not present difference between control groups, however the wound area was higher in the ischemic groups compared to control groups. In the ischemic groups, wound area was smaller in young animals when compared to mature animals. Re-epithelialization was higher in control groups compared to ischemic groups 12 and 14 days after the injury. All groups presented great amount of inflammatory cells. In control group and ischemic groups, younger animals presented thick red-yellowish collagen fibers parallel to surface, while the mature animals presented thin and disorganized red collagen fibers. Ischemic groups presented an increase in amount of blood vessels compared to control groups. Data demonstrated that this model of chronic wound represents a good model for the study of impaired wound repair, being more evident in mature rats.

Keywords: *Aging, Ischemia, Skin, Wound healing*

11.3- MMP2 and MMP9 Expression in Gerbil Female Prostate During the Estrous Cycle

Fernanda Cristina Alcantara dos Santos^{1,5}, Luis Antonio Justulin-Junior², Sabrina Santos Rochel³, Ricardo Alexandre Fochi³, Luiz Roberto Falleiros-Jr¹, Patricia Simone Leite Vilamaior⁴, Sérgio Luis Felisbino², Sebastião Roberto Taboga¹

1. IBILCE/UNESP, Universidade Estadual Paulista, Rua Cristovão Colombo, 2265, Jardim Nazareth, São José do Rio Preto, SP. 2. UNESP, Universidade Estadual Paulista, Departamento de Morfologia, Instituto de Biociências, Botucatu, SP. 3. UNICAMP, Universidade Estadual de Campinas, Departamento de Biologia Celular, Instituto de Biologia, Campinas, SP. 4. UNIRP, Centro Universitário de Rio Preto, Departamento de Biologia, São José do Rio Preto, SP. 5. UFG, Universidade Federal de Goiás, Instituto de Ciências Biológicas, Goiânia, GO.

Hormonal oscillations that occur during the gerbil estrous cycle affect both the structure and function of the female prostate. These alterations include increased prostatic growth during the proestrus and estrus phases and decrease of glandular development in the diestrus I and II. These repeated cycles of glandular development and involution can cause extensive reconstruction and stromal remodeling in the female prostate. Thus, this study evaluated the matrix metalloproteinases (MMPs 2 and 9) expression in the gerbil female prostate during the estrous cycle. Daily vaginal smears were collected at 10:00 am to determine estrous cycle phases. Females were divided into 4 groups (n = 5): proestrus (P), estrus (E), diestrus I (DI), and diestrus II (DII). The female urethras were isolated for dissection of prostate tissue and the blood samples were collected for serological analysis of testosterone, estradiol and progesterone. Histological sections were submitted to immunohistochemistry for the detection of MMP 2 and 9. Serum estradiol levels remained constant through P, DI and DII (33–39 pg/ml) before reaching their maximum concentration peak during the E phase (49.6 ± 15 pg/ml). The progesterone levels were higher during the DII phase (19.08 ± 10.77 ng/ml), and

remained constant during the other phases (10.4–12.8 ng/ml). The serum testosterone levels were similar during P, DI and DII (0.31–0.41 ng/ml), and slightly higher in the E (0.48 ± 0.09 ng/ml). The immunohistochemical analysis showed that higher expression of MMP 2 and 9 occurs during the more hormonal fluctuation of estradiol and progesterone (E and DII, respectively). The MMP2 showed high epithelial and stromal expression during the DI and DII. Conversely, MMP9 showed high epithelial and stromal expression during E, which is the stage of highest prostate development. MMP2 and 9 are the most studied enzymes because of their ability in degrading various elements of the extracellular matrix such as collagens I, III and IV, fibronectin and elastin. In E, the estradiol peak appears to be related to the glandular development and to MMP9 activity in the stroma reshape expansion. Moreover, high expression of MMP2 is mainly in DII, which is a stage marked by high progesterone levels and intense stromal remodeling that occurs during female prostate involution. Thus, these results support a role of these enzymes in modulating the structure and function of gerbil female prostate during estrous cycle.

Keywords: *estrous cycle, female prostate, immunohistochemistry, matrix metalloproteinases, steroids hormones*

11.4- Changes in Bladder of Rats Pre-Puberty as a Result of High Doses of Corticosterone Simulating Estrsse Chronic. Stereological and Morphometric Analysis

Gisele Ribeiro, Suelen Freitas Costa

2. IBRAG-UERJ, Laboratório de Pesquisa Urogenital- Unidade IBRAG- UERJ, Av. 28 de Setembro, 87, HUPE- Vila Isabel- Rio de Janeiro- RJ. 20551-030

Introduction: Stress causes the activation of hipotalamo-hipofise-adrenal axis (HHA), which interferes in hipotalamo-hipofise-gonadal axis (HHG). Clinical trials and experiments involving humans showed the side effects of glucocorticoids (GC) excess in testis production of testosterone. Prolonged treatment with high doses of glucocorticoids simulates in organism a chronic stress condition and a long exposition to any stressing factor causes additional reactions such as increase of GC releasing. **Objective:** To evaluate stress effect in pre pubertal Wistar rats bladder caused by chronic treatment with corticoid. **Material and methods:** Ten new born Wistar male rats divided into 2 groups, control and treated which was given a daily corticosterone intraperitoneal injection (schedule: 9-10 am) (2 mg/100g body weight), since the 7th day of life till sexual maturity, marked by testis descending (25th day). Animals were killed in the 65th day and the bladder was removed, fixed in formalin buffered, processed and embedded in paraffin. Sections of 5 μ m were stained with: Masson's Trichromic, to evaluate vessels and to measure the epithelium, Weigert's resorcin fuchsin, to analyze elastic system fibers and Picro Sirius red for qualitative analysis of collagen types. Stereology method was used to evaluate all the parameters with the Image J software. A biochemical hidroxiprolin assay was performed to quantify the total collagen. Statistical analyses were done using test t student (significance $p < 0.05$). **Results:** There was a decrease of elastic system fibers ($p < 0.0008$) and blood vessels ($p < 0.0006$) in treated group. Picro Sirius red under polarized light suggests predominance of collagen III in treated group and collagen I in control group.

The other parameters didn't show statistical difference between the groups. *Conclusion:* Stereological analysis showed that stress caused by chronic treatment with high doses of corticosterone in pre pubertal phase decrease elastic system fibers and number of vessels per area in rats bladder, however it didn't show any alteration in total collagen.

Keywords: *Bladder, Glucocorticoids, Stress*

11.5- Collagen I and III Location in the Normal and Doxazosin-Treated Rat Prostate

Flávia Karina Delella^{1,2}, Sérgio Luis Felisbino²

1. UNICAMP, State University of Campinas, Cidade Universitária Zeferino Vaz, Monteiro Lobato, 255 - Campinas/SP/Brazil. 2. UNESP, Sao Paulo State University, Department of Morphology, Institute of Biosciences, Botucatu/SP/Brazil

Doxazosin, an alpha-adrenoceptor antagonist, induces the relaxation of smooth muscle cell tonus and reduces the clinical symptoms of the benign prostatic hyperplasia (BPH). However, the doxazosin effects in the prostate stromal microenvironment are not fully known. In a previous study, we showed that doxazosin treatment increases the elastic fibers deposition in the three rat prostatic lobes. Herein, we investigated the effects of doxazosin on types I and III collagen fibers deposition in the stroma compartment from ventral, dorsal and anterior rat prostatic lobes, comparing the results with the control group. Intact adult Wistar rats (n=5) or adult Wistar rats (n=5) treated with doxazosin (25mg/kg/day) during 30 days were killed by an overdose of pentobarbital and the ventral, dorsal and anterior prostates were excised. The prostates samples were frozen in liquid nitrogen and processed for frozen section immunohistochemistry for type I collagen (Genetex, GTX 26308, lot. 16425) and type III collagen (Abcam, ab 6310-100, lot. 424315). The immunohistochemistry showed that types III collagen fibers are widely distributed in the prostate stroma, mainly close to the epithelium and epithelial infoldings, organized bordering the smooth muscle cells and in the interstitium, while type I collagen predominated in the interstitium in the three prostatic lobes. Doxazosin-treatment increased the amount of both collagen fibers types in the three rat prostatic lobes, with a major collagen deposition in the anterior prostate than the other lobes. Morphologically, both fibers became denser and waved, comparing with the fibers found in the control prostatic lobes. In conclusion, the smooth muscle cells contraction blockade by doxazosin may promote changes in prostatic tissue mechanical demand, which is counterbalanced by collagen fibers deposition around cells. This stromal remodeling should be considered in the BPH long-term treatment by alpha-blockers and further studies are need to investigate if this changes can interfere on the prostate epithelium-stroma interactions.

Keywords: *Collagen Fibers, Doxazosin, Immunohistochemistry, Prostate, Stroma*

11.6- The Decrease Proteoglicans and Increase Elastin and Fibrilin Content in Low-Intensity Physical Activity in Aorta from Wistar-Kyoto and Spontaneously Hypertensive Rats

*Jessica Teixeira, Alyne Fonseca, Carlos Alberto Mandarim-de-Lacerda,
Jorge José de Carvalho*

1. UERJ, Universidade do Estado do Rio de Janeiro, Avenida 28 de setembro, numero 87 Fundos

Studies of spontaneous hypertension in rats have focused on the presence of sympathetic hyperactivity and the mechanisms by which this alteration contributes for changing mean blood pressure and the structure and function of arterioles. Nowadays, hypertension is mainly considered as a cardiovascular risk factor, and more attention has been focused on the structure and function of hypertensive arteries, which greatly influence the development of complications in hypertensive vascular disease. Different proportions of elastin and microfibrils promote variable functional characteristics adaptable to the tissue local needs. The ECM is critical for tissue remodeling after an injury or in a disease). It is well established that elastin and collagen content is increased in hypertensive patients and in genetic or experimentally induced hypertension in animals. It is well known that exercise training can reduce blood pressure, with simultaneous improvement in endothelial-dependent vasodilatation. Thus, our aim is to investigate with more accurate tools if exercise training alters the behavior of fibers of the elastic system in the aorta of genetically hypertensive rats . 32 male rats with two-month-old were allocated into 4 groups: sedentary WKY (WKY-SED), exercised WKY (WKY-EX), sedentary SHR (SHR-SED), and exercised SHR (SHR-EX). Sedentary rats were restricted to cage-bound activity, while exercised ones underwent a 1 h/day, 5 days/week exercise training; the experiment last 20 weeks. Desired final training intensity and duration (16m/min, 0% grade, 60 min/day). Exercise training was characterized as low-training since O₂ consumption did not exceed 55% of O₂ max throughout experiment. Blood pressure was measured weekly (BP). Ultrastructural appearance and elastic fiber system were investigated. Results: BP increased progressively in SED-SHRs reaching 189±4 mmHg, but exercise training impaired this process. Final BP in EX-SHR was similar to WKY rats (118±4 vs. 114±4 mmHg, respectively). Exercised groups had lower proteoglicans expression than respective sedentary groups. Percentage of elastic fibers was lower in SED-SHR compared to SED-WKY, but exercise training increased them. Elastin and fibrilin immunostain were seldom seen in SED-SHR, but both EX-WKY and EX-SHR groups showed higher amount of stain. Conclusion: Low-intensity exercise training increases elastin and fibrilin and decrease de proteoglicans content in aorta wall from WKY and SHR rats.

Keywords: *aorta, elastin, fibrilin, physical activity, spontaneously hypertensive rats*

11.7- Remodeling of Extracellular Matrix in the Carotid Body of Hypertensive Rats Induced By L-Name

Alyne Fonseca, vinicius Rocha, Beatriz Barbosa, Jorge Jose de Carvalho

1. UERJ, universidade do estado do rio de janeiro, avenida 28 de setembro 87, fundos

The high prevalence of hypertension and subsequent increased risk of cardiovascular diseases including acute myocardial infarction, the stroke and chronic renal failure, high blood pressure poses a principal risk factor for mortality worldwide. Among non-pharmacological therapies for hypertension, the changes in lifestyle, such as reduced body weight, restriction of salt in the diet, restriction of alcohol consumption and physical activity. In this context, evidence about that the reduction of respiratory rate by use of electronic devices that interact with the patient, advising him to breathe in more slowly, decreases blood pressure in patients with mild and moderate hypertension and those with resistant hypertension and without changes in the use of medication. In front of the bifurcation of common carotid artery is the carotid body (CC), structure sensitive to changes in arterial blood gases and concentration of hydrogen ions. The parenchymal cells of the CC detect changes of these gases. They are activated when the PO₂ decreases and when the PCO₂ and / or [H⁺] increase, and responds with increased releasing neurotransmitters (especially catecholamines), causing a hyperventilation to restore the concentration of these gases in the blood. The present work aims to study the remodeling of the extracellular matrix of carotid body of animals with induced hypertension (induced by L-NAME, a nitric oxide synthase inhibitor) and compared to the carotid body of animals with normal. 14 male rats (250-300g) were divided into two groups (control and L-NAME). The L-NAME group, was administered 40mg/kg/dia in drink water for 45 days. The body mass and blood pressure were followed. The carotid body was removed along with the bifurcation of the carotid artery and processed for optical microscopy. Were analysed the cell nucleus, proteoglycans and collagen 1 and 3. In L-name group, there was an increase of proteoglycans between the cell glomus regarding control group. The carotid body appears less preserved, decrease the number of type 1 collagen fibers and increase the expression of type 3 collagen. Moreover, there is a discontinuity of the capsule around the body. In conclusion, the hypertension induced by L-NAME increased the expression of proteoglycans and collagen type 3, suggesting that hypertension causes a remodeling of the extracellular matrix in the carotid body

Keywords: *carotid body, hypertension, L-name, remodeling*

11.8- *Mycobacterium Leprae* Modulates TGF-Beta1 Expression in Schwann Cell Line

Rafael Braga Petito, Thaís Porto Amadeu, Tércia Rodrigues Alves, Maria da Graça Araújo Baruque, Elizabeth Pereira Sampaio, Euzenir Nunes Sarno

1. IOC, Instituto Oswaldo Cruz, Av. Brasil, 4365, Rio de Janeiro, RJ

Neural fibrosis is one of the causes of deformities during leprosy, which remains a major global health problem. In the progress of neural degeneration, as suggested by others, the Schwann cells (SC) are involved in the degeneration and regeneration of the nerve. Some studies have shown that *Mycobacterium leprae* (ML) induces TGFβ1 production, and its high levels were detected on leprosy. Since TGFβ1 is important in fibrosis development, the aim

of this study was to evaluate the possible role of ML and TGF β 1 on the Schwannoma cell line (ST88-14) in leprosy neural lesion context. ST88-14 cells were stimulated with or without ML for 1, 3, 6, 24, 72 hours and 7 days to evaluate TGF β 1 levels, its mRNA (PCR), its intracellular protein and its receptor (TGFRII) expression (flow cytometry). This cytokine levels were measured (ELISA) in these supernatants cultures. Our results showed that ML stimulation induced a rapid increase in TGF β 1 mRNA expression, followed by a decline that maintained until 6 hours. This cytokine secretion seems to accompany mRNA expression. However, after 6 hours, TGF β 1 secretion seems to return to baseline levels and to rise again after 7 days. TGF β 1 intracellular expression also seems to accompany cytokine secretion. However, after 6 hours, TGF β 1 intracellular levels seems to return to increase and reach its maximum at 48 hours and, after 72 hours, to return to baseline levels again after 7 days. ML also up regulated the TGFRII expression. These partial results suggest the involvement of ML in Schwann cell line (ST88-14) answer modulation to TGF β 1, and their contribution in neural fibrosis. Supported by CNPq, CAPES, FAPERJ, FIOCRUZ.

Keywords: *LEPROSY, Mycobacterium leprae, SCHWANN CELL, TGF-BETA1*

11.9- An In Situ Study of the Effect of Metallo and Serine Proteinases Inhibitors on the Birefringence of the Secretory-Stage Enamel Organic Extracellular Matrix

*Alexandre Ribeiro do Espírito Santo^{2,1}, Marcelo Rocha Marques¹,
Sérgio Roberto Peres Line¹*

1. FOP-UNICAMP, Faculdade de Odontologia da UNICAMP, Av. Limeira, 901, Areião, CEP 13414903, Caixa Postal 52, Piracicaba-SP, Brasil. 2. ICS-UFBA, Instituto de Ciências da Saúde da UFBA, Av. Reitor Miguel Calmon, S/N, Vale do Canela, CEP 40110902, Salvador-BA, Brasil

Dental enamel formation occurs extracellularly and the establishment of an ordered enamel organic extracellular matrix (ECM) seems to be a crucial step for proper construction of the enamel mineral phase. Polarizing microscopy shows that the ordered supramolecular structure of the secretory-stage enamel organic ECM exhibits strong birefringence. We have previously reported that this birefringence is lost in unfixed specimens, probably due to extensive proteolytic cleavage of enamel proteins. Therefore, the present work aimed at investigating the association between enamel proteinases' activities by analyzing the effects of metallo and serine proteinases inhibitors in situ on the birefringence of the secretory-stage enamel organic ECM. Male Wistar rats weighing $\approx$ 200 g were used in the present study. After sacrifice by cervical displacement, distal 10 mm fragments of upper incisors were immediately removed and immersed for 15 h under continuous shaking, at 37° C, in one of the following solutions: 1. 10 mM Tris pH 8.0, 150 mM NaCl (NEGATIVE CONTROL, n=8); 2. 2% paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS), pH 7.2 (POSITIVE CONTROL, n=5); 3. 10 mM Tris pH 8.0, 150 mM NaCl, 2 mM 1,10-phenanthroline (n=9); 4. 10 mM Tris pH 8.0, 150 mM NaCl, 2 mM phenylmethylsulphonyl fluoride (PMSF) (n=8); 5. 10 mM Tris pH 8.0, 150 mM NaCl, 2 mM 1,10-phenanthroline, 2 mM PMSF (n=9). Samples were then immersed in proper fixative

solution for 24 h and submitted to laboratorial procedures which allowed obtaining 5- μ m-thick longitudinal sections of the secretory-stage enamel organic ECM. These sections were immersed in 80% glycerin for 30 min and analyzed by transmitted polarizing light microscopy. 1,10-phenanthroline (inhibitor of metalloproteinases) and 1,10-phenanthroline + PMSF (inhibitor of serine proteinases) clearly prevented decrease in the optical retardation of birefringent brightness from the tissue referred to. PMSF alone promoted a slight preservation of the birefringence exhibited by the secretory-stage enamel organic ECM. Concluding, rapid loss of birefringence in not immediately fixed secretory-stage enamel organic ECM is caused by the activity of enamel proteinases and the activity of metalloproteinases seems to lead to a preliminary degradation of the enamel organic ECM, which in turns probably facilitates subsequent serine proteinases activity. Support: Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP.

Keywords: *Enamel, Birefringence, Enamel proteinases, PMSF, Phenanthroline*

11.10- Low-Dose of Celecoxib Accelerates Cutaneous Wound Healing Delayed by High-Fat Diet in Obesity Prone and Resistant Rats

Adriana Paulino do Nascimento, Andréa Monte Alto Costa

1. UERJ, UNIVERSIDADE DO ESTADO DO RIO DE JANEIRO, Rua Professor Manoel de Abreu, 444 3 andar Vila Isabel Rio de Janeiro

Obesity is a problem of epidemic proportions and is associated with increased incidence of chronic wounds. Nonsteroidal anti-inflammatory drugs have been widely used to diminish the inflammatory reaction, through the inhibition of cyclooxygenase enzyme. Our aim was to study the effects of high-fat diet on cutaneous wound healing of obesity prone and resistant rats and to investigate the effects of two doses of celecoxib about these conditions. Wistar rats were fed either with a high-fat (n=39) or a standard diet (n=18, control, C) for 20 weeks, when the diet group was subdivided in obesity prone (n=20, OP) and resistant (n=19, OR) groups, an excisional lesion was done, and the animals were killed 7 days later. Treated groups received a daily dose of celecoxib 5 or 10 mg/kg/day from two days before wounding until euthanasia. Wound contraction, re-epithelialization, body mass and retroperitoneal fat were evaluated. After euthanasia, lesion and adjacent normal skin were formol-fixed and paraffin-embedded. Granulation tissue was analyzed and hydroxyproline assay were realized. Body mass was higher in OP ($p<0.001$) than other groups and retroperitoneal fat was different between all groups, being higher in OP ($p<0.001$) and smaller in C group ($p<0.01$ versus OR). Animals fed high-fat diet presented delayed wound contraction ($p<0.001$). OP and OR groups presented higher amount of inflammatory cells than C group, being mast cells in OP ($p<0.01$ versus C; $p<0.05$ versus OR) and polymorphonuclears in OR ($p<0.05$). The density of myofibroblasts in OP and OR groups was reduced compared with C group ($p<0.01$ and $p<0.001$, respectively). Collagen fibers density and hydroxyproline levels were higher in OP group ($p<0.05$) compared with other groups. In groups treated with celecoxib we observed a reduction in body mass in OP treated with 5 mg ($p<0.001$) and 10 mg ($p<0.01$), without alteration in percentage of retroperitoneal fat. Wound contraction was delayed in C group treated with 10 mg of celecoxib ($p<0.001$) and this dose didn't alter the wound contraction in

OP and OR. However, 5 mg of celecoxib accelerates wound contraction in OP ($p<0.001$) and OR ($p<0.01$) groups to the level of control group. This study showed that high-fat diet exerts negative effects on rats cutaneous wound healing, in obesity prone as well as in obesity resistant animals, due mainly to prolongation of the inflammatory phase and that a low-dose of celecoxib accelerates cutaneous repair in these conditions.

Keywords: *Celecoxib, High-fat diet, Skin, Wound healing*

11.11- Effects of Androgen and Estrogen Suppression on the Prostatic Stroma of Old Gerbils

Silvana Gisele Pegorin de Campos^{1,3}, Daniele Lisboa Ribeiro¹, Wellerson Rodrigo Scarano², Rejane Maira Goes¹, Sebastião Roberto Taboga¹

1. IBILCE/UNESP, São Paulo State University, R Cristovão Colombo 2265, 15054-000 São José do Rio Preto, SP, Brazil. 2. UNIFAL, Federal University of Alfenas, R Gabriel Monteiro da Silva, 700, 37130-000, Alfenas, MG, Brazil. 3. HCB, Barretos Cancer Hospital, R Antenor Duarte Villela, 1331, 14784-400, Barretos, SP, Brazil

The aim of this study was to evaluate changes in prostatic stroma of old gerbils (18 months) after surgical castration associated or not to steroidal blockade. The animals were divided in 6 groups and all of them were surgically castrated, excluding the control composed by intact animals. The other two groups were formed by castrated animals and castrated that received the drug vehicle. In the experimental groups doses of flutamide (antiandrogen, 10mg/kg/day) and/or tamoxifen (antiestrogen, 1mg/kg/48h) during 1, 3, 7 (short-term) and 30 (long-term) days post-castration were applied. The methodologies involved: morphological (HE, Gömöri reticulin, Gömöri trichrome), immunohistochemical analysis (chondroitin sulphate, type IV collagen) and quantitative and statistic evaluation. Proteoglycans were identified at transmission electron microscopy after fixation with Cuprolinic Blue. The castration associated or not to anti-steroidal drugs by short or long-term promoted alterations in the distribution of stromal cells and of extracellular matrix elements of the prostate. Gradually, the epithelial compartment, more representatives in intact animals, was significantly exceeded in volume by the stromal compartment, characterizing a gland atrophy process. The smooth muscle cell (SMC) frequency increased significantly after 30 days of therapy in experimental and control vehicle groups, and this component had an effective participation to the increase of the stroma in the experiment end. Large collagen I and chondroitin sulphate deposits in the subepithelial region was a hallmark of prostatic acini in the experimental groups up to 7 days. Additionally, in regions of epithelial projections into the acinar lumen was also observed a large of type IV collagen deposition. Two populations of prostatic stromal proteoglycans were identified associated with basement membrane based on their morphology and dimensions and designated P1 (31-54 nm) and P4 (83-179 nm). These elements appeared in the form of filaments and were modulated by treatments performed. The response of the prostatic stroma front of hormonal ablation suggested that the gland architecture is often related to changes in extracellular matrix and SMC, and this showed specific reactions to short-and long-term therapy. Furthermore, this study seems to be

a pioneer in the use of steroid blockers in old animals and that is looking closer to the period in which humans have higher prostate cancer rates. Support: FAPESP

Keywords: *castration, old gerbil, prostatic stroma, proteoglycans, smooth muscle cell*

11.12- Remodeling of Extracellular Matrix in the Left Atrioventricular Valve in Hypertensive Rats

Vinícius Novaes Rocha, Rodrigo Neto Ferreira, Carlos Alberto Mandarin de Lacerda, Jorge José de Carvalho

1. UERJ, Universidade Estadual do Rio de Janeiro, Av 28 de Setembro,
47 fundos - 3º andar

The heart valves are designed to maintain the unidirectional blood flow and for this reason they have an anisotropic architecture of the extracellular matrix that ensures and maintains proper function under conditions of high and low pressure. The valve disease is a disorder that can affect any of the heart valves, marked by any change in its structure that prevents its proper operation. Studies show that the mechanical stress caused by blood flow may interfere with the organizational structure of cells and extracellular matrix of the valve. This study aimed to investigate the possible structural and ultrastructural changes that hypertension, induced by L-NAME, can cause to the left atrioventricular valve (mitral) from hearts of rats. The animals received L-NAME (40mg/kg/dia), previously diluted in water, for five weeks. At the end of the fifth week euthanasia of animals was performed and the parietal leaflet fragments were collected and processed for light microscopy and electron microscopy. It was analyzed the morphological structure of the valves at both light microscopy and electron microscopy, as well as the thickness of the valve and quantification (% / area) of the elastic and collagen fibers through the ImagePro-Plus 4.5. The animals treated with L-NAME developed hypertension from the second week of administration of the drug. Structurally, it was possible to identify a disorganization of the elastic and collagen fibers with regions of fragmentation. The animals with hypertension showed thicker valve regions than control, plus a smaller amount of collagen fibers. Ultrastructurally, it was identified changes of the endothelium in animals treated with L-NAME, where it presented irregular contours and accumulations of vesicles inside. Furthermore, it was also observed fragmentation of basal lamina, disorientation of collagen fibers, accumulation of oxytalan fiber near the endothelium and fragmentation of elastic fibers of the atrial layer. This study concluded that increased blood pressure, characterized by hypertension, causes changes in the morphological structure of the valve, which may be linked in with the synergistic inhibition of nitric oxide synthesis, and may thus undermine its perfect operation.

Keywords: *Extracellular Matrix, Hipertension, L-NAME, Valve*

11.13- Microfibril-Elastin Fiber System Distribution in the Rat Mitral Valve: A Structural and Ultrastructural Study

Vinícius Novaes Rocha, Rodrigo Neto Ferreira, Carlos Alberto Mandarim de Lacerda, Jorge José de Carvalho

1. UERJ, Universidade do Estado do Rio de Janeiro, Av. 28 de setembro,
47 fundos - 3º andar

With increasing valve disease tissue-engineered heart valves are the ultimate solution for treating these conditions. Healthy heart valves maintain unidirectional blood flow and the anisotropic matrix architecture assures sustained and adequate functioning under high-pressure conditions that remodel in response to changes in local mechanical forces. A major challenge in tissue engineering is determining the tissue structures similar to heart valve anatomy and function. The essential extracellular mitral valve components include glycoproteins, collagen and elastin. However, little is known about the elaunin and oxytalan fibers which together with the elastic fibers form a system with elastic characteristics associated with a common and sequential ontogenetic development. The microfibril-elastin fiber system was studied to investigate the interrelationship of oxytalan, elaunin and elastic fibers in mitral valve morphology. The valves were stained with orcinol-neo fuchsin and Weigert's resorcin-fuchsin with or without prior oxidation and observed under light microscopy. Routine electron microscope was used to study valve samples for elastic, elaunin and oxytalan fibers which were fixed in 2.5 % glutaraldehyde. For elastin and fibrillin immunostaining for electron microscopy, the tissues were gradually infiltrated in LR WHITE resin and the ultrathin sections were incubated in primary antibody to highlight fibrillin-1 and elastin. In the valve, the existence of the fibers of different diameters was differentiated by staining affinities and in transmission electron microscope was observed that the microfibril-elastin fiber system was distributed in different proportions in the mitral valve layers. In atrial layer, elastic fibers predominated, characterized by large amounts of elastin. The spongiosa layer was composed mainly by elaunin fibers formed by microfibrils interspersed with elongated patches of amorphous material, and oxytalan fibers characterized by large bundles of microfibrils without elastin. In ventricular layer there were elaunin fibers arranged in continuous bundles oriented parallel to endothelium. The present study suggesting that the microfibril-elastin fibers play a role in the mechanical protection and maintenance of the integrity of the mitral valve.

Keywords: *Elastic fibers, Elaunin fibers, Oxytalan fibers, Mitral valve*

11.14- Schwann Cell Transdifferentiation: Possible Role of Myofibroblast in Neural Fibrosis During Leprosy

Rafael Braga Petito, Thaís Amadeu, Tercia Alves, Sergio Antunes, Elizabeth Sampaio, Euzenir Sarno

1. IOC/FIOCRUZ, Instituto Oswaldo Cruz, Av Brasil 4365

The development of deformities during leprosy remains a major global health problem. Fibrosis has been seen in leprosy nerve biopsies of patients and Schwann cell (SC) seems to be involved in nerve degeneration and regeneration. Our group showed, in a human Schwannoma lineage (ST88-14), increased mRNA levels of TGF β 1 and its receptors after *Mycobacterium leprae* (ML) stimulus. Since TGF β 1 is important to fibrosis development, and promotes transdifferentiation of SC into myofibroblast, the aim of this study was: a) to analyze α actin smooth muscle expression (α SMA, myofibroblastic marker) in patients nerve biopsies without pathological changes or with fibrosis, and b) to evaluate *in vitro* SC-myofibroblastic transdifferentiation and extracellular matrix (ECM) secretion/deposition. α SMA expression was analyzed in patients' nerve biopsies by immunohistochemistry and immunofluorescence. In order to evaluate, *in vitro*, SC-myofibroblastic transdifferentiation (immunofluorescence and flow cytometry to α SMA) and ECM (collagen IV, laminin, fibronectin and tenascin) secretion/deposition and proteins structural form (ELISA and immunofluorescence, respectively), human primary Schwann cells and ST88-14 cultures were stimulated with recombinant human TGF β 1 (10 ng/ml) for 24 hours, 4 or 7 days. Our results showed, for the first time, α SMA expression in perineurium of normal nerves tissues and in some endoneurial cells of nerve tissues with fibrosis. After 7 days of stimulation, an increased expression of collagen IV, laminin, fibronectin and tenascin, and, in some cases, the structural form modulation of these proteins were also observed. Moreover, TGF β 1 induced transdifferentiation of SCs into myofibroblasts. The α SMA intracellular increased after 24 hours, 4 and 7 days of stimulation. These results suggest a TGF β 1 involvement in SC-myofibroblastic transdifferentiation, which can contribute to fibrosis and, consequently, to neural degeneration. Supported by CNPq, CAPES, FAPERJ, FIOCRUZ.

Keywords: *Leprosy, Schwann cell, Myofibroblast, Fibrosis, Extracellular matrix*

11.15- Effect of Oral Supplementation of L-Glutamine on the Rats Ventral Prostate Subjected to Abdominal Irradiation

Flávia Cristina Morone Pinto, Pamella Campos Silva, Carolina dos Santos Silva, Waldemar Silva Costa

1. UERJ, Universidade do Estado do Rio de Janeiro, Rio de Janeiro

PURPOSE: To analyze the effects of L-glutamine on the rats ventral prostate submitted to radiotherapy. **METHODS:** Wistar rats, approximately 90 days and 350g, were subjected to pelvic irradiation with or without nutritional supplementation of the amino acid L-glutamine, divided into three groups: (1) Control (n=5), (2) group subjected to irradiation abdominal (n=5), without amino acid supplementation, (3) group submitted to abdominal irradiation with supplementation of L-glutamine (n=5). Supplementation of L-glutamine (0.65g/kg body weight) by gavage occurred during the 21 days of experiment. Pelvic irradiation was used for the single dose of 1000cGy. on the 8th day of experimentation. Euthanasia of the three groups occurred in the 22 th day of the experiment. After the ventral prostate was removed and fixed by drying. The morphometric analysis was conducted for the area of the prostate acini and height of the acinar epithelium. According to the area, the acini were classified into three groups: small (with area up to 10,000 μ m²), average (with area greater than 10,000 μ m² and

im2 less than 100,000) and large (with an area exceeding 100,000 im2). Statistical analysis: Kruskal-Wallis followed by Mann-Whitney for non-parametric analysis of data; chi-square for the analysis of proportions and percentages. In all cases the differences were considered statistically significant when $p < 0.05$. RESULTS: In the supplemented group had a significant weight gain of weight (+14.1g) when compared to the control (+20.8 g) and irradiated (3.1 g). There was significant reduction in acini classified as small in the supplemented group (6.52%) compared to control (35.23%), the Spent (12.84%). In contrast there was an increase of acini average (76.94%) in the group receiving supplementation, as compared to the control (43.24%) to irradiated (55.23). Referring to large acini, the proportion was higher in the irradiated group (31.93). There was a significant decrease in the height of the acinar epithelium in groups 2 (44.28 nm) and 3 (31.88 nm) compared to 1 (66.55 nm). The distribution of collagen was higher in group 3 (9.13%) than in group 1 (8.14%) and 2 (7.0%). CONCLUSION: Irradiation changed the distribution of acini, the acinar epithelium of the characteristics and distribution of collagen per field. And so far it is believed that L-glutamine has a role in protection/restoration of the ventral prostate. Keywords: L-glutamine, Radiotherapy, Rat Financial Support: FAPERJ, CNPq

Keywords: *L-glutamine, Radiotherapy, Rat*

11.16- Elastogenesis in the Development of the Interpubic Ligament During Mouse Pregnancy

Sílvia Roberto Consonni¹, Cláudio Chrysostomo Werneck², Camila Fernandes Moro¹, Renata Giardini Rosa¹, Lygia da Veiga Pereira³, Robert P Mecham⁴, Paulo Pinto Joazeiro¹

1. Unicamp, State University of Campinas, Department of Histology and Embryology. 2. Unicamp, State University of Campinas, Department of Biochemistry. 3. USP, University of Sao Paulo, Department of Genetics and Evolutionary Biology. 4. WUSTL, Washington University School of Medicine, Department of Cell Biology and Physiology

The organization of elastic fibers involves the synthesis and the deposition of molecules in a highly regulated sequence to ensure the elastic characteristics of each organ in the early stages of development. Pelvic tissues, which are extremely rich in elastic fibers, remodel during pregnancy as does the birth channel in mammals. This remodeling is essential to the vaginal delivery and it occurs mainly in the uterus, cervix and vagina. In mouse and guinea pig, the pubic symphysis articulation also remodels in a controlled hormonal process which allows safe delivery. This phenomenon comprises the “transformation” of the fibrocartilage into an interpubic ligament (IpL) followed by its relaxation before parturition. After the first parturition, morphological aspects of the interpubic articulation become similar to that which is observed in nulliparous mouse. Recently, prolapse and pelvic organ support impairment had been described in genetically modified mouse for the proteins involved in the elastogenesis as lisil oxidase-like 1 and fibulin-5 at postpartum and fibulin-3 in aged mice. Since, ligaments are the main supportive structure of pelvic organs, the initial aim of this work was to evaluate the elastogenesis in the development of the IpL during mouse pregnancy, due to its association with pathologies in the mouse pelvic organ support. Thus virgin and pregnant C57Bl/06 female mice groups were studied using light and transmission

electron microscopy. In virgin and 12th day of pregnancy (D12), the fibrocartilaginous tissue had slender bundles of microfibrils randomly distributed among the fibrochondrocytes. Weigert's Resorcin-Fuchsin stain and ultrastructural studies revealed the presence of immature elastic fibers (IEF), which consist of small conglomerates of amorphous material, distributed among the bundles of microfibrils in the development of the IpL at D15. In the D18, the IpL showed IEF and all tissue compounds aligned to the opening axis of the articulation. So, the induction of the elastin deposition on the microfibrils of the developing ligament could contribute to the elasticity necessary to the pelvic girdle in the period of preparation for delivery. This process that occurred during the adult life has unique characteristics of a model that could be used to understand the fiber elastic formation. Still, further analyses will be done using a fibrillin-1 deficient mouse that recapitulates the vascular aspect of Marfan syndrome. Fapesp: 08/56492-0

Keywords: *Pubic symphysis, Biology of reproduction, Elastogenesis, Extracellular matrix, Mouse*

11.17- Matrix Metalloproteinases, Timps and Growth Factors Regulating Ameloblastoma Behaviour

Adriane Sousa de Siqueira¹, Márcia Regina Dias Carvalho², Ana Celina Dourado Monteiro², Vanessa Moraes Freitas¹, Ruy Gastaldoni Jaeger¹, João de Jesus Viana Pinheiro^{2,1}

1. USP, UNIVERSITY OF SAO PAULO, . Prof. Lineu Prestes, 1524 Ed. Biomedicas 1.
2. UFPA, FEDERAL UNIVERSITY OF PARA, Rua Augusto Corrêa, 01 - Guamá. CEP 66075-110. Caixa postal 479

Ameloblastoma is an odontogenic neoplasm with local invasiveness and recurrence. We previously suggested that growth factors and matrix metalloproteinases (MMPs) influence ameloblastoma local invasiveness (Pinheiro et al, 2004). Here we studied expression of MMPs, TIMPs, and growth factors in ameloblastoma. Correlation analysis assessed relationships among these molecules. Furthermore, we searched for cell signalling markers in ameloblastoma. Immunohistochemistry detected MMPs, TIMPs, and growth factors in formalin-fixed paraffin-embedded samples of human ameloblastoma. Immunohistochemical staining was quantitatively assessed by three independent observers (A.S.S., M.R.D.C., A.C.D.M.), with a minimal inter observer variability (>5%). Sections were analysed using light microscopy, at a final magnification of 400x. Both neoplastic and stromal cells were evaluated. Expression of different antigens was scored according to number of cells with positive staining, without considering differences in staining intensity. Labelling index (LI = positive cells/total cells x 100) for MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, EGFR, EGF and TGF-alpha was determined by observing 1000 cells. All ameloblastoma samples presented MMPs 1, 2 and 9; TIMPs 1 and 2; and EGF, TGF-alpha and EGFR. LI of MMP-9 and TIMP-2 was equally distributed in neoplastic cells and stroma. In ameloblastoma cells MMP-1 and MMP-2 expression was significantly higher compared to stroma. EGF and TGF-alpha exhibited no differences between neoplastic cells and stroma. EGFR LI was increased in ameloblastoma cells compared to stroma. We found positive correlation between EGF and

TIMP-1; and between TGF- α and TIMP-2. Immunohistochemistry detected ERK 1/2 and phospho-ERK in ameloblastoma. Phospho-ERK was mostly detected in stroma. Results suggest an interplay involving growth factors, MMPs and TIMPs, which may contribute to ameloblastoma behaviour. Signals generated by this molecular network would be transduced by ERK 1/2 pathway.

Keywords: *Ameloblastoma, Extracellular Matrix, Growth Factors, Metalloproteinases, TIMPs*

11.18- Effect of Low Intensity Laser Therapy Irradiation on Skin Tissue Repair

Alan Lopes de Oliveira¹, Vanessa Araújo da Silva¹, Oscar Guimarães¹, Mauro Geller^{1,2}, Adenilson de Souza da Fonseca^{1,3,4}, Severo de Paoli¹, Flávia de Paoli¹

1. UNIFESO, Centro Universitário Serra dos Órgãos, Avenida Alberto Torres, 111 Alto.
2. UFRJ, Universidade Federal do Rio de Janeiro, Ilha do Fundão. 3. UERJ, Universidade Estadual do Rio de Janeiro, Rua 7 de setembro Vila Isabel. 4. UNIRIO, Universidade Federal do Estado do Rio de Janeiro, Rua Frei Caneca Centro

Low intensity laser therapy is a form of phototherapy used to promote wound healing in different clinical conditions. Laser therapy at an adequate wavelength, intensity, and dose can accelerate tissue repair. However, there is still conflicting information about the effect of multiple irradiations on the cellular responses of wounded cells. To better understand the use of low intensity laser therapy in cutaneous wound healing, clinical studies that correlate cellular effects and biologic processes are needed. The aim of this study was evaluated the effect of low intensity laser therapy on the wound healing process in rats skin. Five Wistar rats were subjected to a surgical incision measuring 6cm in length, located to right side to the spinal column and sutured in U-shape. A surgical incision on the left side was done, sutured and used to control. The treated incisions were irradiated with a 658 nm laser, with the following parameters: 2.5 Hz, 3 min, and 4 J/cm². Skin biopsy was performed on the 7, 14 and 21 days after wounding, for morphological evaluation of the cicatricial process. Morphological analysis was performed with an optical microscope (Olympus, EUA) adapted to a computerized picture analysis system to capture image (Image Proplus 6). Results indicated that effect of lower doses (4 J/cm²) determines the stimulatory effect, showed complete re-epithelization around 14 days after surgical incision; non-irradiated incision was re-epithelized around 21 days. In addition, fibroblasts and inflammatory cells were observed in treated and non-treated incisions after 7 days of the surgical procedure, decreased in 14 and 21 days. We concluded that low intensity laser therapy can accelerate skin wound healing.

Key words: *laser, skin, wound healing*

11.19- Effects of Metoclopramide-Induced Hyperprolactinemia on the Gene Expression of Hyaluronan Synthases I, II and III in Mouse Uterine Along the Different Phases of the Estrous Cycle

Regina Célia Teixeira Gomes¹, Carina Verna¹, Ana Isabel Malva Silva¹, Fernanda Lopes de Freitas Condi Condi², Ricardo dos Santos Simões², Helena Bonciani Nader¹, José Maria Soares Júnior Soares Júnior¹

1. UNIFESP, Universidade Federal de São Paulo, Rua: Botucatu, nº 740, 2º Andar, Ed. Lemos Torres. 2. USP, Universidade de São Paulo, Rua: Dr. Arnaldo, nº 455

OBJECTIVE: To evaluate effects of metoclopramide-induced hyperprolactinemia on the gene expression of hyaluronan synthases I, II and III in mouse uterine along the different phases of the estrous cycle. **DESIGN:** Experimental study using an semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) method for examined the expression pattern of hyaluronan synthase (HAS I, II and III) mRNAs in the mouse uterine. **ANIMAL(S):** 80 adult (100 days old) female virgin mice. **METHODS:** The animals were randomly divided into two groups of 10 animals each: control (Ctr), which received the vehicle (0.2 ml NaCl 0.9% in distilled water) and experimental (HPrl), which received 200 µg metoclopramide dissolved in 0.2 ml NaCl 0.9% in distilled water. The medications and the vehicle were administered subcutaneously once daily at 10 a.m. for 50 consecutive days. In the 50th day, one hour after the last drug or vehicle injection, a vaginal smears evaluation was performed in order to verify the estrous cycle phase. **INTERVENTION(S):** Soon thereafter the animals were sacrificed and properly separated into 8 subgroups: proestrus (Ctr) e (HPrl), estrus (Ctr) e (HPrl), metaestrus (Ctr) e (HPrl) and diestrus (Ctr) e (HPrl). Following euthanasia, the uterine horns were removed, sectioned and immediately frozen in liquid nitrogen for RNA extraction to detect tissue (HASI, II and III) by Polymerase Chain Reaction (RT-PCR). Blood was collected for the dosage of prolactin and serum estrogen and progesterone using ELISA-like assay. The results were expressed as mean $\pm$ SD and were analyzed by the Student's t-test. Significance level was set at $P < 0.05$. **RESULTS:** The HAS I, II and III were gene expressed of the metoclopramide-treated group was superior to those of experimental group during the proestrous, estrous and diestrous. Hormonal serum levels were as follows: estradiol (pg/mL), Ctr = 21 ± 0.816 vs. HPrl = 30 ± 0.923 ($P < 0.001$); progesterone (ng/mL), Ctr = 9.67 ± 0.077 vs. HPrl = 7.01 ± 0.031 ($P < 0.001$); and prolactin (ng/mL), Ctr = 4.1 ± 0.082 vs. HPrl = 6.8 ± 0.134 ($P < 0.001$). **CONCLUSION:** Our results suggest that metoclopramide-induced hyperprolactinemia may affect the gene expressed on female mice uterus during all estrous phases. Possibly, this change may help to understand the infertility problems related to high levels of prolactin. Peripherally, the serum levels of progesterone were reduced and those of estradiol were increased.

Keywords: *Hyaluronan Synthase, Hyperprolactinemia, Prolactin, Uterus*

11.20- L-Glutamine and L-Arginine Protective Effect in Corpus Cavernosus of Rats Submitted to Pelvic Irradiation

Jorge Luiz Medeiros Junior, Bruno Felix Patricio, Ligia Silva Ponde Serra, Monica Nunes Prazeres Ribeiro, Flávia Cristina Morone Pinto, Luiz Eduardo Macedo Cardoso, Francisco José Barcellos Sampaio, Waldemar Silva Costa

1. UERJ, Universidade Estadual do Rio de Janeiro, Rio de Janeiro

Introduction: Radiotherapy is commonly used in cancer treatment affecting pelvic organs next to target organ. Many alterations can be observed in pelvic radiation treatment, such as: erectile dysfunctions and urinary morbidity. To prevent this problem different radioprotectors drugs have been studied. **Objective:** To evaluate the protector action of L- arginine and L- glutamine in erectile tissues submitted to pelvic radiation. **Material and methods:** It was used 35 Wistar rats penis, the rats aged between 3 and 4 months. Animals of control group (C) were maintained under standard laboratory conditions, while animals belonging to the other groups, IA and IG, were submitted to pelvic radiotherapy on the 8th day of the experiment. Animals were irradiated with a single dose of 1,164 cGy applied to the pelvis, been killed 7 (I-7, IA-7 e IG-7) and 15 (I-15, IA-15 e IG15) days after irradiation. The amino acids were administered once a day at the dose of 0.65 g/kg per day, starting 7 days before the irradiation until the last day of experiment. Animals from control and irradiated groups received 5 ml of water similarly administered once a day throughout all the experiment. Histochemical techniques were performed to stereological analysis of smooth muscle, collagen and elastic system fibers of corpus cavernosum. Image J software was used to quantify the corpus cavernosum area with 100 points grid. For qualitative analysis different types of collagen it was used Picro Sirius red under polarized light. Statistical analysis was performed by one way ANOVA and by Bonferroni post-test. The level of significance was set at ($P < 0.05$). **Results:** I-15 group showed a significant increase in collagen amount ($p < 0.004$). Smooth muscle increased in I-7, IG-7, IA-7, IA-15 and IG-15 groups ($p < 0.05$). Elastic system fibers increased in I-15 and IG-15 ($p < 0.05$) when compared to control and IA-15 groups. Qualitative analysis of collagen, showed modifications in different types of collagen disposition, in I-7 group prevalence of green color propose a bigger disposition of type III collagen, while I-15 group has a bigger incidence of red color, when compared to I-7 group. Similar alterations happened between IG-15 and IA-15. **Conclusion:** L-arginine seems to protect the corpus cavernosum. L-glutamine prevent collagen alteration, but didn't act in elastic system fibers recovery. Both amino acids seem do not present a protective effect in relation to smooth muscle.

Keywords: *Radiation, L-glutamine, L-arginine, Rat*

11.21- Maternal Protein Restriction Induces Fibrosis in Rat Ventral Prostate of Male Offspring

Jaqueline de Carvalho Rinaldi, Sergio Alexandre Alcantara dos Santos, Livia Maria Lacorte, Carolina Sarobo, Luis Antonio Justulin Junior, Sergio Luis Felisbino

1. UNESP, Department of Morphology, IB - Sao Paulo State University, District of Rubiao Jr, Botucatu-SP/Brazil

Fetal programming has been associated with impairment of fetal development and cardiovascular disease. Recent studies have associated fetal programming by in utero protein restriction with delay in sexual maturation in male rats. Thus, the aim of this study was to investigate the effects of fetal programming induced by in utero protein restriction on the collagen fibers content in the rat ventral prostate. Male Wistar rats with underwent in utero fetal programming by protein restriction (normal diet=17% protein; protein in restricted diet=6%) was killed at age of 16 week and the ventral prostate was excised, weighted and processed for histochemistry and immunohistochemistry for type I and III collagens. The programmed male offspring presented decrease in both absolute and relative ventral prostate weight compared to matched control. The histochemistry reaction showed that programmed prostates presented increased stromal space with stronger immunostaining for both type I and III collagens, mainly in the interstitial space, than control prostates. The programmed prostates also exhibited a decrease in parenchymal volume fraction compared to age matched control. In conclusion, fetal programming induced by in utero protein restriction lead to a prostatic fibrosis associated with impairment in parenchyma compartment maturation.

Keywords: collagen fibers, fetal programming, prostate, protein restriction

11.22- Effect of Testosterone on the MMP-2 and Timp-2 Expression in the Gerbil Male and Female Prostates

Sabrina Santos Rochel^{3,1}, Fernanda Cristina Alcantara Santos¹, Patricia Simone Leite Vilamaior⁴, Sérgio Luís Felisbino², Sebastião Roberto Taboga¹

1. Ibilce/UNESP, Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto – SP. 2. IB/UNESP, Instituto de Biociências, Botucatu – SP. 3. IB/UNICAMP, Instituto de Biologia, Campinas – SP. 4. UNIRP, Centro Universitário de Rio Preto, São José do Rio Preto - SP

Matrix Metalloproteinases (MMPs) are enzymes that participate in the process of Extracellular Matrix (ECM) remodeling and are regulated by a set of glycoproteins called Tissue Inhibitors of Metalloproteinases (TIMPs). MMPs and TIMPs are known to play a role in normal physiological processes and pathological states. An imbalance between TIMPs and MMPs rate may participate in tumor progress in several tissues, including the prostate. Morphological evidences point to a similarity between the ventral lobe of the male prostate and the female prostate of the gerbil *Meriones unguiculatus*. Thus, this work presents the distribution of MMP-2 and TIMP-2 in both gerbil female prostate and ventral lobe of male prostate, and the effect of Testosterone (T) over these enzymes. 5 intact males and 5 intact females in the proestrus phase were kept as control groups (Con). Experimental groups consisted of 10 males submitted to orchietomy for 7 and 21 days (groups 7C and 21C respectively, n=5) and 10 females injected with Testosterone Cipionate (5mg/kg/48h) during 7 and 21 days (groups 7T and 21T respectively, n=5). Histological sections of prostate tissues were submitted to immunohistochemistry for the detection of MMP-2 and TIMP-2. The male

ventral prostate of Con showed marked expression of active MMP-2, mainly in the blood plasma, and its expression seemed to increase with the ablation of T, being more intense in 21C blood vessels and fibroblasts. Conversely, immunostaining for TIMP-2 occurred in the epithelial cells of control males and decreased with castration, being positive mainly in blood plasma in the 21C group. In the intact female prostates, expression of active form of MMP-2 occurred in epithelial cells, secretion, blood plasma and stromal fibroblasts. In 7T group there was a reduction in the activity of these enzymes, showing weak stain in the secretion, fibroblasts and few secretory cells. On contrary, 21T group showed intense expression of MMP-2, especially in basal membrane and prostatic secretion. Epithelial cells of control female prostates were TIMP-2 positive but in the 7T and 21T groups its expression decreased, with positive reaction only in border of apical cells. The results demonstrate that either the lack of androgen in males or the excess of T in females promote an imbalance in MMP/TIMP rate, increasing the MMP-2 expression, which is related to the process of ECM remodeling that occur in the development of pathologies. Proc. FAPESP 2006/06985-5

Keywords: *Female prostate, Gerbil, MMP, TIMP, Ventral prostate*

11.23- Doxazosin Decreases MMP-2 and MMP-9 Activity in Rat Ventral Prostate

Larissa Mayumi Ribeiro, Jaqueline de Carvalho Rinaldi, Livia Maria Lacorte, Wellerson Rodrigo Scarano, Luis Antonio Justulin Junior, Sérgio Luis Felisbino

1. UNESP, Instituto de Biociências, Universidade Estadual Paulista, Rubião Junior, S/N, Cx Postal: 510, CEP: 18.618-000 - Botucatu / SP

Doxazosin (Dox), a quinazoline-derived α 1-adrenoceptor antagonists has been found to induce apoptosis in prostate epithelia cells and to suppress the formation of prostate tumors and the metastasis of prostate cancer in the TRAMP model. Recently, new studies have demonstrated that Dox also downregulate expression of AR and PSA in vitro. Here, we investigated the effect of Dox on activity of MMP-2 and MMP-9, enzymes related to the extracellular matrix remodeling and prostate cancer invasiveness. Adult male Wistar rats were treated with daily doses of doxazosin (25mg/kg), dissolved in corn oil and subcutaneous injected. Age matched animals, receiving only the vehicle, were used as control. After 3 and 30 days of treatment, a group of six animals were killed with sodium pentobarbital overdoses. The ventral prostatic (VP) lobe was excised and immediately weighed and processed to histology or snapped frozen in liquid nitrogen and processed to gelatin-zymography. Picrosirius analyzes demonstrated that Dox treatment increased collagen volume fraction in the prostatic estroma. The activity of both MMP-2 and MMP-9 was reduced after 3 and 30 days of Dox treatment. These results demonstrate that Dox treatment results in fibrosis and changes activity of extracellular matrix-remodeling enzymes.

Keywords: *collagen, doxazosin, MMPs, prostate*

11.24- Stromal Remodelation and Metalloproteinases (MMP2 And MMP9) Activity in Prostatic Chronic Inflammation from Adult Rats Exposed to Di-N-Butyl-Phthalate (DBP) During Gestation and Lactation

Wellerson Rodrigo Scarano^{1,2}, Fabíola Choqueta de Toledo⁵, Marina Trevizan Guerra⁵, Silvana Gisele Pegorin de Campos⁴, Luis Antonio Justulin Jr.², Sebastião Roberto Taboga³, Sérgio Luis Felisbino², Wilma De Grava Kempinas²

1. UNIFAL-MG, Universidade Federal de Alfenas, Rua Gabriel Monteiro da Silva, 700, Alfenas, MG. 2. UNESP, Instituto de Biociências de Botucatu, Distrito de Rubião Júnior, s/n., Botucatu, SP. 3. UNESP, IBILCE, São José do Rio Preto, SP. 4. Hospital do Câncer, Fundação Pio XII, Barretos, SP. 5. UNICAMP, Programa de Pós-Graduação em Biologia Celular e Estrutural, Campinas, SP.

People are constantly exposed to environmental contaminants from industrial processes, through air, food, water or contact with a variety of consumer products. Di- γ -butyl-phthalate (DBP) is widely used as a plasticizer in many products including medical devices, flexible plastics and some cosmetic formulations. In the present study we evaluated the toxic effects on the male adult rat prostate of DBP exposure during fetal and lactational periods, because although many studies have addressed the influence of phthalates on the male reproductive system, only a few have discussed their possible effects on prostate development. Pregnant females were distributed into two experimental groups: Control (C) and Treated (T). The females of the T group received DBP (100 mg/Kg, by gavage) from gestation day 12 to postnatal day 21, while C rats received the vehicle (corn oil). In adulthood (90 days old), the animals were euthanized. The serum and testicular testosterone levels were measured. Ventral prostate was removed and weighed. Distal segment fragments of the ventral prostate were fixed and processed for histochemistry (HandE and Gömöri reticulin). Protein extraction from ventral prostate fragments was performed for Gelatin zymography for MMP-2 and MMP-9 (MMP, metalloproteinase). Stereological and histopathological analyses were also performed. Serum and testicular testosterone levels and prostate weight were comparable between groups. In the T group the relative proportions (%) of epithelial (C=32.86; T=42.04*) and stromal (C=21.61; T=27.88*) compartments were increased, while the luminal compartment was decreased (C=45.54; T=30.08*), *p<0.05. Increase of collagen fibers among the acini, which enlarged the space among the glandular portions, was found in the T group. This aspect corroborated the stereological analyses that showed elevation in the relative stromal volume in these animals. Furthermore, in T animals, mononuclear inflammatory infiltrated areas were observed and, a fibrillar rearrangement occurred with formation of collagen bunches dispersed among the inflammatory cells while in some areas the collagen and reticular fibers appeared to support the infiltrate under the epithelium. The gelatinolytic activity of MMP9 was more intense in the T than the C group, while the MMP2 activity was similar between the groups. Furthermore, the elevated MMP9 activity observed was involved in the fibrillar degradation and rearrangement caused by inflammatory processes.

Keywords: prostate, metalloproteinases, collagen fibers, reticular fibers, inflammation

11.25- Rotational Stress Delays Cutaneous Wound Healing

Bruna Romana de Souza, Andréa Monte Alto Costa

1. UERJ, State University of Rio de Janeiro, Rua prof Manoel de Abreu, 444, 3º andar, 20550-170, Rio de Janeiro, RJ, Brazil

Objective: To evaluate macro and microscopically the effects of the rotational stress on cutaneous wound healing in mice. **Method and Results:** Male Swiss mice aged 8-12 weeks (25-30 g) were housed five per plastic cage and chronically submitted to stress environment. Rotational stress was applied to the animals by spinning the cages at 45 rpm for 15 minutes every hour three days before wounding and was continued daily until euthanasia. Control animals (n=5) were not submitted to rotational stress. A full-thickness excisional lesion (1 cm²) was created. Wound area was measured soon after wounding and 3 and 7 days after and the animals were killed 7 days after wounding. Lesions and adjacent skin were formalin-fixed and paraffin-embedded. Sections were stained with hematoxylin-eosin and toluidine blue, and immunostained for F4/80, alpha-smooth muscle actin and proliferating cell nuclear antigen (PCNA). Furthermore, the myeloperoxidase (MPO) activity was measured on wound area. Data are reported as mean $\pm$ standard error of mean. The wound area was smaller in the control group than in the stress group 3 and 7 days after wounding ($p < 0.05$ and $p < 0.01$, respectively). Seven days after wounding, the number of PCNA-positive connective tissue cells was greater in the control group than in the stress group ($p < 0.001$). Furthermore, the MPO activity and the number of F4/80-positive macrophages and mast cells were greater in the control group than in the stress group 7 days after wounding ($p < 0.0001$, $p < 0.05$ and $p < 0.0001$, respectively). Nevertheless, the number of blood vessels and myofibroblast density were smaller in the control group than in the stress group 7 days after wounding. **Conclusions:** Rotational stress impairs mice cutaneous wound healing by reducing the wound closure, cellular proliferation and inflammatory cells migration and increasing the angiogenesis and myofibroblastic differentiation. **Financial support:** Centro de Produção da Universidade do Estado do Rio de Janeiro (CEPUERJ) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

Keywords: *cutaneous wound healing, psychological stress, collagen fibers, inflammatory cells, myofibroblasts*

11.26- Intrauterine Androgen Exposure Promotes Epithelial and Stromal Disorders in the Adult Mongolian Gerbil Male and Female Prostate

Manoel Francisco Biancardi¹, Fernanda Cristina Alcântara dos Santos², Ana Paula Silva Perez¹, Rejane Maira Góes², Sabrina Santos Rochel¹, Luiz Roberto Falleiros-Jr², Patrícia Simone Leite Vilamaior³, Sebastião Roberto Taboga²

1. UNICAMP, UNIVERSIDADE ESTADUAL DE CAMPINAS, PPGBCE, Avenida Bertrand Russel, s/n, CEP 13083-865 Campinas, SP. 2. UNESP/IBILCE, UNIVERSIDADE ESTADUAL PAULISTA, Rua Cristóvão Colombo, 2265, Jd Nazareth, CEP: 15054-000, São José do Rio Preto. 3. UNIRP, Centro Universitário de Rio Preto, Rua Yvette Gabriel Atique, 45, Boa Vista, CEP 15025-400, São José do Rio Preto

Some researches have demonstrated that exposure to endocrine-disrupting chemicals, like androgens, during embryonic phase is associated with the appearance of benign and malignant prostatic lesions on adult and aging life. Moreover, alterations on the normal development of female phenotype like the malformation of external genitalia, appearance of seminal vesicle and early death may happen. In this manner, the aim of this work was to study the effect of the intra-uterine exposure to testosterone on prostatic embryogenesis of gerbil (*Meriones unguiculatus*) females and males. Thus, the prostatic glands of the adult animals including normal and those treated in gestation (day 18^o) with testosterone cypionate (1mg) were processed for light microscopy. The total blood of them was collected for serologic analyses of testosterone, estrogen and progesterone levels. Histological sections were stained with hematoxylin-eosin and Gömöri reticulin for general studies. Immunohistochemistry was performed for α -actin. The results showed that female exposed to androgens on gestation have high hormonal levels for all hormones, while in the treated males only progesterone presented elevated values when comparing with control animals. The morphological analyses showed that the both male and female controls have prostatic glands of typical standard, with acini lined in simple cylindrical epithelial, a lumen rich in secretion and stroma composed mainly by fibroblasts and smooth muscle cells. However, the animals exposed to androgens during embryonic period showed many alterations in prostatic gland. In both male and female was possible identify some inflammation foci in association with prostatic intraepithelial neoplasia (PIN). Throughout immunohistochemistry for α -actin it was possible to identify some areas of smooth muscle layer interruption caused by epithelial cells proliferation into glandular stroma. These results showed severe alterations in the prostatic glands of the animals exposed to testosterone during prostatic embryogenesis, showing the potential that endocrine-disrupting agents may have on imprint of some reproductive tract organs. Taking into account it is important to know the action mechanisms of these disruptors and understand the factors that determinate the different process of prostatic morphogenesis in males and females, as well as those that can predispose this gland to develop malignant lesions during adult and aging life.

Keywords: *Androgen exposure, Intrauterine, Mongolian Gerbil, Prostate, Prostatic embryogenesis*

11.27- Morphological Analysis of Wound Healing in the Skin of Wistar Rats Treated with Marigold

*Vanessa Araújo da Silva*¹, *Alan Lopes de Oliveira*¹, *Oscar Guimarães*¹, *Mauro Geller*^{1,2}, *Adenilson de Souza da Fonseca*^{1,3,4}, *Severo de Paoli*¹, *Flávia de Paoli*¹

1. UNIFESO, Centro Universitário Serra dos Órgãos, Rua Alberto Torres, 111. 2. UFRJ, Universidade Federal do Rio de Janeiro, Ilha do Fundão. 3. UERJ, Universidade do Estado do Rio de Janeiro, Rua 28 de setembro Vila Isabel. 4. UNIRIO, Universidade Federal do Estado do Rio de Janeiro, Centro

Calendula officinalis (marigold) has been widely used in homeopathic medicine for the treatment of many diseases. It has been reported to possess many pharmacological activities, which include antioxidant anti-inflammatory antibacterial, antifungal and antiviral. It also

possesses cytotoxic as well as tumor reducing potential. The aim of this study was to evaluate the scarring capability of marigold on experimental skin wounds in Wistar rats. Each animal was subjected to a surgical incision measuring 6cm in length, located to right side to the spinal column and sutured in U-shape. Marigold extract (10 mg) was placed on the incisions for 7, 14 and 21 days, and no treatment was provided to the left side (control). Skin biopsy was performed on 7, 14 and 21 days after wounding, for morphological evaluation of the cicatricial process. Morphological analysis was performed with an optical microscope (Olympus, EUA) adapted to a computerized picture analysis system to capture image (Image Proplus 6). Results showed that marigold allowed the highest growth in cells directly involved in the cicatricial process, as fibroblasts and inflammatory cells, when compared to control animals. Control animals showed complete re-epithelization around 21 days after surgical incision while treated animals had reduced this period of time to 14 days. Our finds suggest that marigold possesses a potent wound healing activity. Biological activity could be related to pharmacological activities of this product.

Keywords: *calendula officinalis*, skin, wound healing

11.28- Immunolocalization of SLRPS in the Pregnant Endometrium of Decorin-Deficient Mice

Juliane Sanches, Telma Zorn, Sérgio Oliveira

1. USP, University of São Paulo, 05508-900. São Paulo, Brazil

Changes in collagen fibrillogenesis have been previously detected in the pregnant mouse endometrium, where very thick collagen fibrils are present exclusively in the decidualized stroma. Fibrillogenesis is a complex process that involves several steps, including association with other extracellular molecules, such as proteoglycans. The small leucine-rich proteoglycans decorin, biglycan and lumican are known to play an important role in this process. The present study is an investigation into the distribution of biglycan and lumican in the pregnant endometrium of wild-type (Dcn^{+/+}) and decorin-deficient mice (Dcn^{-/-}). Methods: Immunoperoxidase staining was performed on days 3 and 7 of pregnancy in the decorin-null (Dcn^{-/-}) and wild-type (Dcn^{+/+}) mice. The samples were fixed in 4% paraformaldehyde, incubated with rabbit polyclonal antibodies raised against murine biglycan (LF-106) (Fisher et al. 1995) and lumican (Chakravarti 2000). Peroxidase was visualized using 3, 3'-diaminobenzidine (Sigma) in PBS with 0.03% H₂O₂. The sections were counterstained with Mayer's hematoxylin. Results: Our results demonstrate that on day 3 of pregnancy, biglycan was expressed exclusively around bundles of smooth muscle cells of the myometrial external layer in Dcn^{+/+} animals. In contrast, in Dcn^{-/-} animals biglycan was detected in the uterine stroma and glandular epithelium, and was maintained in the external layer of the myometrium. Lumican immunostaining, however, was intense in the decidualized and non decidualized stroma from Dcn^{-/-} animals on day 7 of pregnancy being absent in the Dcn^{+/+} animals, lumican in either decidualized or nondecidualized stroma. Together with previous results from our group, these results suggest that decorin, biglycan and lumican play an interactive role on collagen fibrillogenesis in the mouse endometrium, which is modulated according to the stage of pregnancy.

Key words: *decidua, collagen, decorin-deficient*

11.29- Evaluation of Markers of Oxidative Stress in Ischemic and Non-Ischemic Skin Lesions

Tamires da Silva Santos¹, Samuel Santos Valença³, Andréa Monte ALto Costa¹

1. UERJ, Universidade do Estado do Rio de Janeiro, Av. Professor Manoel de Abreu, 87 - Vila Isabel - Rio de Janeiro - RJ - Cep 2055. 2. UERJ, Universidade do Estado do Rio de Janeiro, Av. Professor Manoel de Abreu, 87 - Vila Isabel - Rio de Janeiro - RJ - Cep 2055. 3. UFRJ, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, Bloco K, 2º andar, sala 35, Cidade Universitária

A large proportion of the population is affected by problems of skin healing, but the mechanisms that cause these disorders in wound healing are not well known. Studies suggest oxidative stress as one of the possible causes of these problems. The objective of this project is to evaluate the redox balance in Wistar rats comparing ischemic and non-ischemic wounds. Wistar rats were divided into two groups: control (n = 7) and ischemia (n = 7). Under anesthesia, in d0, an excisional non- ischemic wound (1 cm²) was performed in the control group. In ischemic group, two bilateral incisions measuring 7 cm separated by a distance of 3 cm were performed, the skin was separated from the subjacent tissue and the incisions were sutured; then an excisional wound (1 cm²) was performed between the incisions. Wound contraction was evaluated macroscopically. Euthanasia was performed seven days after wounding. The wound and normal skin were removed, homogenized and biochemical analyses to antioxidant enzyme activities and biomarkers of oxidative damage were evaluated. The activity of glutathione peroxidase was lower in ischemic wound when compared to non-ischemic wound (p <0.05). The catalase and superoxide dismutase activities were lower in the ischemic wound compared to normal skin (p <0.001). The glutathione/oxidized glutathione ratio, a marker of oxidative stress was reduced in the ischemic wound as compared to non-ischemic wound (p <0.01). The myeloperoxidase activity was increased in ischemic wound when compared to non ischemic wound (p<0.001). Nitrite level was higher in ischemic wound when compared to non-ischemic wound (p<0.01). Our result indicates a redox imbalance suggesting oxidative stress in non-ischemic wounds that may be delay the wound healing.

Keywords: *skin, wound ischemic, wound non-ischemic, wound healing, oxidative stress*

11.30- Effects of Cyclooxygenase Blockade on Cutaneous Wound Healing Depends on Gender

Jeanine Salles dos Santos, Andréa Monte Alto Costa

1. UERJ, UNIVERSIDADE DO ESTADO DO RIO DE JANEIRO, Rua Professor Manoel de Abreu, 444 3 andar Vila Isabel Rio de Janeiro

A substantial body of work describes the contrasting influences of androgenic and estrogenic sex steroids on the healing of acute skin wounds. These endogenous sex steroid hormones profoundly influence the response to cutaneous injury. The aim of this study was to investigate if cyclooxygenase blockade affects cutaneous wound healing in the same way in both genders. Forty adult male and female (20 males and 20 females) BALB/c mice (3 months; 20 – 25 g) were used and divided in two groups in each gender: one control and one treated. Treated group received a daily dose of aspirin 100 mg/kg/day dissolved in saline, i.p. and the control group received the same volume of saline, i.p.. The treatment started two days before wounding and extended for fourteen days after wounding until euthanasia, when wounds with adjacent skin were removed. The wounds consisted in a full-thickness excisional wound (1 cm²) in the dorsal surface. Sections obtained were stained with hematoxylin-eosin and Sirius red. Immunohistochemistry against α -smooth muscle actin was also performed. In both male and female treated groups the wound contraction and the neo-epidermis formation were delayed when compared to the control group, but the female group presented a more expressive delay. The inflammatory infiltrate was abundant in both male and females treated groups, being also more pronounced in the female group. Concerning collagen density, both male and females treated groups presented smaller density of these fibers when compared to the control group. In female group collagen density was also smaller than the male group as well as myofibroblastic differentiation. In conclusion, it was showed that cyclooxygenase pathway inhibition, by high dose of aspirin, affected wound healing process delaying wound contraction, neopidermis formation and granulation tissue formation in both male and female groups, the delay was more expressive in females.

Keywords: *Aspirin, Cyclooxygenase, Gender, Skin, Wound healing*

11.31- The Effects of Diabetes on the Structure and Cell Proliferation of the Myometrium in the Early Pregnancy of Mice

*Rodolfo Favaro Ribeiro, Renato de Mayrinck Salgado, Priscila Ribeiro Raspantini,
Zuleica Bruno Fortes, Telma Maria Tenório Zorn*

1. USP, Universidade de São Paulo, São Paulo

The myometrium is a highly dynamic uterine compartment, which is deeply remodeled along the full course of pregnancy. Four sequential myometrial phenotypic phases were identified during rat pregnancy. The first phase is characterized by hyperplasia of the smooth muscle cells (SMCs). Afterwards, there is a transition phase, followed by a phase of hypertrophy of SMCs and extracellular matrix synthesis. Finally, at the end of pregnancy, myometrial SMCs acquire a contractile phenotype to perform labor. Type 1 diabetes mellitus is associated with elevated rates of preterm birth. However, the mechanisms underlying its causes are hampered by our limited knowledge of the impact of this pathology to the myometrium. The goal of this study was to investigate the effects of diabetes on the myometrium during the first adaptation phase of pregnancy. Diabetes was induced in female Swiss mice by intravenous injection of alloxan (40mg/Kg). Diabetic animals (>400 mg/dl) were mated between 50-70 and 100-120 days after diabetes induction. Pregnant animals were killed at 168 hours after the detection of the vaginal plug. The myometrium was analyzed at

light and electron microscopy and evaluated by histomorphometry. Cell proliferation was determined by the percentage of PCNA-positive nuclei detected by immunohistochemistry. Diabetic mice showed high levels of hyperglycemia, hypoinsulinemia, glycosuria, increased food and water consumption and decreased body weight. No considerable alterations were found 50-70 days after diabetes induction. On the other hand, important alterations in the structural organization and cell proliferation were observed among the diabetic mice 100-120 days after diabetes induction. These alterations were divided in to subgroups, D1 and D2. Compared to the control group, the distance between muscle layers in D1 was augmented and showed signs of edema. Contrarily, in subgroup D2 the distance between layers showed a dramatic reduction. In both D1 and D2, the thickness of the muscle layers was reduced. Supporting the histomorphometric data, cell proliferation was significantly decreased in the internal and external layers of both D1 and D2. Thus, diabetes-induced myometrial alterations are duration-dependent. Variation among the animals may represent different stages of diabetic complications. We suggest that these alterations may be correlated with disorders occurring in pregnancy, such as preterm delivery. Financial Support: FAPESP

Keywords: *myometrium, diabetes, cell proliferation*

11.32- Ovarian Hormone-Regulated Distribution of Decorin and Biglycan in Mouse Uterine Tissues

Renato de Mayrinck Salgado, Rodolfo Favaro Ribeiro, Fernanda Angela Correia Barrence, Telma Maria Tenório Zorn

1. USP, Universidade de São Paulo, São Paulo

The extracellular matrix (ECM) is a complex structure of macromolecules capable of self-assembly, which exert essential biological functions in the tissues. The endometrial ECM plays important roles in the processes of decidualization, embryo implantation and trophoblast cell invasion. Our laboratory has previously demonstrated the differential distribution of four members of the small leucine-rich proteoglycan (SLRP) family during the estrous cycle and early pregnancy. Decorin and biglycan are class I SLRP and share structural homologies. The aim of this work is to analyze the influence of the ovarian sex hormones estrogen (E2) and progesterone (P4) on the distribution of decorin and biglycan in mouse uterine tissues. For that purpose, mice were ovariectomized and after 20 days they were either sacrificed or treated with E2, medroxyprogesterone acetate (MPA) or E2+MPA. After the surgery or last injection, the uteri were removed, fixed in Methacarn and embedded in Paraplast. Immunoperoxidase was performed using anti-decorin and anti-biglycan primary antibodies. After incubation with the secondary antibody and streptavidin-peroxidase, the reaction was visualized with DAB. After ovariectomy, decorin was found only in the deep stroma and external muscle layer, and biglycan was absent from all uterine tissues; in the E2 group strong immunoreaction for decorin was found in the whole stroma and myometrium, whereas biglycan was observed mainly in the region of basement membranes; in the MPA group decorin was present in the deep stroma and myometrium, and biglycan was present in all uterine tissues; finally, in the E2+MPA group immunoreaction for both proteoglycans was observed in the stroma and in the myometrium. In summary, these results show that the

ovarian hormones modulate the distribution of decorin and biglycan in the mouse uterus and may be involved in the kinetics of synthesis, deposition and degradation of ECM molecules in reproductive tissues. Financial Support: FAPESP

Keywords: *uterus, proteoglycans, estrogen, progesterone*

11.33- Bioengineering of Blood Vessels Using Porcine Xenogenic Implants

Sara Spinola Clemente, Leonardo Boldrini, André Marchiori, Eliene Koslowski, Radovan Borojevic, Chistina Maeda Takiya

1. UFRJ, Universidade Federal do Rio de Janeiro, Rua Prof. Paulo Rocco n° 255 Ilha do fundão

Large-diameter vascular grafts have been successfully developed from polymers such as Dacron or e-PTFE. However, it has been difficult to develop small-caliber grafts (inner diameter < 6 mm) because of biological reactions at the blood-material and tissue-material interface, such as acute thrombogenicity, intimal hyperplasia and formation of aneurisms. Autologous vein grafts require a removal of a vein from another part of the body, when available. Synthetic grafts pose long-term health risks and are not successful for small diameter graft applications. Here we developed a mechanically stable vascular graft using intact iliac arteries of pig decellularized by using sodium dodecyl sulfate (SDS). Components of extracellular matrix involved with the mechanical support and elastic properties (elastin, collagen, chondroitin sulfate, laminin and fibronectin) were evaluated, before and after treatment, by biochemical and histochemical methods. Iliac arteries of pig rendered acellular with SDS and has well-preserved extracellular matrix and basement membrane structure. Our results indicate that we were able to produce a vascular graft with optimal cell seeding characteristics. These properties suggest proof of concept for its use as a scaffold for further vascular tissue engineering.

Keywords: *Bioengenharia, Acelularização, SDS, PróteseVascular*

11.34- Chronic Stress Affects Collagen and Muscle Fibers of Penile Corpus Cavernosum of Rats

Camila Marinho Costa Silva; Diogo Benchimol de Souza; Dilson Silva; Célia Martins Cortez; Waldemar Silva Costa e Francisco José Barcellos Sampaio

1. UERJ, Universidade Estadual do Rio de Janeiro, rua prof manoel de abreu 444, 5 andar

Chronic stressful stimuli can challenge tissue homeostasis, due to adverse effects of glucocorticoids. Early exposure to glucocorticoids can accelerate or delay the functional organic maturation, depending on the dose and exposure time. Intense stress has been associated with reduced serum testosterone levels and reduced genital blood flow, but there is no knowledge about its effect on penile tissue. This work aiming to analyze quantitatively the collagen and muscle fibers in penile corpus cavernosum of chronically

stressed rats. Eight Wistar rats were assigned into the stress group (SG) and were submitted to two hours of tube restraint daily, from the 4th to the 9th week of life. Other 7 rats were assigned as control group (CG) and maintained in standard conditions. After this period, animals were sacrificed by anesthetic overdose, blood was collected for testosterone dosage by radioimmunoassay, and penis was grossly dissected. The mid-shaft of the penis was separated and fixed in 10% formaldehyde, being then processed for paraffin embedding and sliced in 5µm. Twenty five fields of corpus cavernosum stained with Masson's trichromic were captured under 200x of magnification from each. A grid of 100 points was overlaid in the figures with ImageJ software for point counting. Points hitting collagen fibers or muscle fibers were separately counted. Student t test was applied to mean comparisons, considering $P < 0.05$ significant. Testosterone serum concentration decreased from $0.19(\pm 0.04)$ ng/ml in the CG to $0.08(\pm 0.01)$ ng/ml in the SG ($P = 0.02$), showing that the stress protocol was effective in reducing testosterone as previously demonstrated. Muscle fibers also decreased from $14.07(\pm 1.4)\%$ (CG) to $8.98(\pm 1.3)\%$ (SG) ($P = 0.02$) and Collagen fibers increased from $53.66(\pm 3.7)\%$ (CG) to $64.47(\pm 0.9)\%$ (SG) ($P = 0.01$). Stress stimuli induced structural changes in the corpus cavernosum of rats suggestive of penile fibrosis, which may play a role in erection dysfunction.

Keywords: *corpo cavernoso, estresse, rato wistar*

11.35- Extract of Jateí-Kaá Affects Extracellular Matrix Components of the Rat Placenta

Andrea Luiza Cunha Laura, Marciana Sanabria, Fabio Feitoza, Daniella Moraes Antunes, Simone Bertozzi S. Vasconcelos, Joao Maximo Siqueira, Luciane Candeloro Portugal

1. UFMS, Universidade Federal de Mato Grosso do Sul, Cidade Universitaria s/n
Departamento de Morfofisiologia cep 79070-900

In the state of Mato Grosso do Sul, *Achyrocline alata*, known as jatei-kaá, is a plant species popularly used in the treatment of uterine and prostatic infections, hernia, stomachache and bronchitis. However, there is no data available about its influence on pregnancy. The placenta is formed by different tissues, such as the labyrinth, which is formed by vascularized mesenchyme covered by trophoblast layers that interact directly with the maternal blood lacunae. The fetal vessels' basal lamina contributes to the exchange of nutrients and metabolites between the mother and her fetus. Moreover, the placenta has been acknowledged as an important regulator of cell behavior, cell migration and cell-cell interactions. The objective of this work was to analyze the expression of the basement membrane molecules collagen IV and laminin in the placenta of rats treated with jatei-kaá extract and whether this component affects the morphology of normal placentas in vivo. For this purpose, twenty pregnant rats were divided into groups A and B, and received the hydro-ethanol extract of jatei-kaá, which was diluted in the water for consumption. Group A received the extract from day 6 to 15 of pregnancy, whereas Group B received it from day 1 to 20 of pregnancy. A control group ingested only filtered water during the whole course of gestation. Two placentas were obtained from each animal in all groups, fixed in Methacarn

and embedded in paraffin. Serial sections were submitted to immunoperoxidase technique for collagen IV and laminin. We observed an increase in the deposition of collagen IV and laminin in the labyrinth of placentas from group B, as well as a reduction in the maternal vascular spaces, when compared with the control group. However, the immunoreaction for both molecules was similar in the placentas from Group A and the control group. Thus, we suggest that the jateí-kaá extract presents time-dependent toxicity for the rat placenta, as the remodeling of extracellular matrix molecules was observed only in the group submitted to this component for a longer period during pregnancy. Further studies will be necessary to evaluate the consequences of these placental alterations for the fetuses.

Keywords: *jateí-káa, placenta, collagen, remodeling*

11.36- Tissue Remodeling of the Rat Placenta under the Effect of the Jateí-Kaá Extract

Andrea Luiza Cunha Laura, Marciana Sanabria, Fabio Feitoza, Daniella Moraes Antunes, Simone Bertozzi de Souza Vasconcelos, João Máximo Siqueira, Luciane Candeloro Portugal

1. UFMS, Universidade Federal de Mato Grosso do Sul, Cidade Universitária, s/n,
Departamento de Morfofisiologia, Cep: 79050-900

Congenital malformations may occur when noxious substances cross the placental barrier. In the state of Mato Grosso do Sul, *Achyrocline alata*, known as jateí-kaá, is a plant species popularly used in the treatment of uterine and prostatic infections, hernia, stomachache and bronchitis. However, there is no data available about its effects on pregnancy. The process of normal placentation is clearly influenced by the composition and organization of the endometrial extracellular matrix (ECM). Moreover, collagens are ECM macromolecules that exert important structural and functional activities in the various tissues. The aim of the present study was to analyze the morphological characteristics and collagen distribution in the placenta of rats treated with jateí-kaá extract during different periods of pregnancy. For this purpose, twenty pregnant rats were divided into groups A and B, and received the hydro-ethanolic extract of jateí-kaá, which was diluted in the water for consumption. Group A received the extract from day 6 to 15 of pregnancy, whereas Group B received it from day 1 to 20 of pregnancy. A control group ingested only filtrated water during the whole course of gestation. Two placentas were obtained from each animal in all groups, fixed in Methacarn and embedded in paraffin. Serial sections were submitted to Haematoxylin-eosin and picosirius staining techniques, as well as immunofluorescence for collagen I. In all placentas from group B and some from group A, there was a remarkable inflammatory process in the peripheral tissue, especially around the basal decidua and the Reichert's membrane. The picosirius-stained sections showed that in groups A and B, there was an increase in the general collagen content in the chorionic plate and the labyrinth. To confirm that, immunofluorescence for collagen I showed a strong immunoreaction in these same placental regions, being more intense in Group B. Thus, we suggest that the jateí-kaá extract presents time-dependent toxicity for the rat placenta, as the remodeling of collagens of

the ECM was more conspicuous in the group submitted to this component for a longer period during pregnancy.

Keywords: *Placenta, jatei-káa, collagen type I, Inflammation, extracellular matrix*

11.37- The Protective Effect of *Sebastiania hispídum* Aqueous Extract and Low Level Laser Irradiation (LLLI) Against Local Effects of Bothrops Moojeni Snake Venom

Doroty Dourado, Rosemary Matias, Ana Paula Machado, Maria Inês Jardim, Daniel Pereira, Maria Alice Hofling

1. UNIDERP, Universidade para o Desenvolvimento do Estado e da Região do, R. Ceará 333 B. Miguel Couto Campo Grande/MS

The leaves extract of *Sebastiania hispídum* (Euphorbiaceae) have been used in folk medicine. Low level laser irradiation (LLLI) has been shown to accelerate healing in a number of affected tissues. Here, we compared the phytotherapy with LLLI to neutralize the local muscle effects caused by i.m. gastrocnemius injection of *Bothrops moojeni* crude venom (40 µg/mL) in Wistar rats. Three groups of animals were used (n = 4 each): Control, saline-injected (SS); Venom-injected (i.p. 40 µg/mL) (V); Venom-injected + aqueous extract of *S. hispídum* (VSh); Venom-injected + 40 seg irradiation with Indium-Gallium-Aluminium Phosphide (InGaAlP), 660nm, 4 joules/cm² (VL) daily. At 3, 24 and 48h post-venom, blood samples were collected for evaluation of creatine kinase (CK) serum levels, used as marker of muscle fiber damage. After anaesthesia, the animals were killed, and gastrocnemius processed for evaluation of muscle fiber damage and measure of connective tissue deposition. Evaluation of the body weight gain indicated significant difference after 48 h post-envenoming between VL and VSh (353 ± 5.8 and 226.7 ± 5.8). The mean ± SD showed that plasma CK (U/L) levels were significantly different higher in V group compared to control. In all time intervals there was significant decrease in CK release in groups treated with LLLI or VSh compared to Venom group. The morphological damage was attenuated both in animals treated with the plant extract and lasertherapy, with laser irradiation giving better results than the *S. hispídum* (p<0.05). Bothropic venoms produce local muscle damage with fast evolution. Serum therapy is efficacious in treating systemic effects but is ineffective to treat local damage. Alternative non-invasive therapeutic measures such as the described here can be useful in bothropic accidents. Apoio: Anhanguera/UNIDERP, CPP, CNPq, UNICAMP

Keywords: *snake venom, Phytotherapy, Laser therapy, CK, histopatology*

11.38- Metal-Substituted Cytochrome C And Microperoxidases Microinjection Promote the Lost of Cellular Adhesion and Induce Apoptosis in Vascular Smooth Muscle Cells

Tatiana Prieto⁴, Soraya Soubhi Smaili¹, Otaciro Rangel do Nascimento⁴, Ivarne Luis dos Santos Tersariol², Iseli Lourenço Nantes³

1. UNIFESP, Universidade Federal de São Paulo, V Clementino S Paulo. 2. Univ Mogi das Cruzes, Universidade de Mogi das Cruzes, Mogi das Cruzes, SP. 3. Univ Fed ABC, Universidade Federal do ABC, ABC SP. 4. USP, Universidade de São Paulo, São Paulo

Adhesion-mediated apoptosis resistance is an emerging concept that may explain the observed differences in survival cells. Tumor cells interacting either with a specific extracellular matrix protein substrate or with each other or with non-transformed cells, such as fibroblasts, exhibit increased resistance towards a wide variety of therapeutic approaches. Cytochrome *c* is a mitochondrial respiratory hemeprotein that contributes for apoptosis triggering when it is released from mitochondria as well as it is exogenously deliver to cells via microinjection or carriers. In the present study, apoptosis was induced by delivery to cells of exogenous metal-substituted cytochrome *c* and microperoxidases. The deliver of the agents of cell death was done by cellular microinjection in rabbit aortic smooth muscle cells. It was observed that only zinc-substituted cytochrome *c* that retains the native cytochrome *c* structural features was efficient to activate caspase-3 and consequently apoptosis with lost of cellular adhesion. The free base, Fe³⁺, Mn³⁺ microperoxidases-11 and free base- cytochrome *c* led to occurrence of typical events of the apoptosis program, such as: nuclear fragmentation, membrane blebbing and extracellular exposure of phosphatidylserine, independently of caspase-3 activation. We also observed that cellular treatment with zinc-substituted cytochrome *c* as well as with native cytochrome *c* lead to lose of cellular adhesion triggering cell death. These results show that the lost of cellular adhesion and consequently induction of cell death in vascular smooth muscle cells is independently of caspase-3 activation. Supported by FAPESP, CNPq and FAEP-UMC.

Keywords: *cytochrome C, microperoxidases, cellular adhesion, apoptosis, smooth muscle cells*

ÍNDICE REMISSIVO (PRIMEIRO AUTOR) INDEX (1ST AUTHOR)

Abreu, EO, 19
Accardo, CM, 24
Almeida, JC, 28
Almeida, MES, 12
Alves, J, 7
Amaral, RJFC, 41
Araujo, APB, 31, 31

Atayde, SA, 18, 25
Augusto, TM, 7
Baldo, C, 23
Barbosa, FAL, 34
Barbosa, GO, 26
Belmiro,CLR, 2
Beraldo, FH, 30

Berardo, PT, 25
Biancardi, MF, 50
Bittencourt, DA, 14
Borbely, AU, 23
Borges, LF, 25
Borsonello, N, 16
Braga, FFR, 4
Brand, C, 15
Brohem, CA, 32
Calvet, CM, 20
Campos, L, 22
Campos, SGP, 46
Cândido, EM, 6
Cardeal, LBS, 35
Cardoso, AB, 26
Carvalho, LCC, 4
Carvalho, SN, 41
Cascabulho, CM, 15
Castro, VCLW, 29
Clemente, SS, 52
Consonni, SR., 47
Cronemberger, VS, 27
Cruz, KSP, 37
Cunha, G, 1
De Aro, AA, 3, 4
Deccache, PMS, 21
Delella, FK, 44
Della Colleta, HHM, 12
Dezonne, RS, 28
Di Gangi, R, 20
Dourado, D,
53Dreyfuss, JL, 22
Eller, CM, 29
Espírito Santo, AR, 45
Estofolete, CF, 34
Felsemburgh, F, 9
Ferreira, AK, 38
Ferreira, JG, 36
Ferreira, JG, 36
Figueiredo, CC, 34
Florim, RK, 23
Fonseca, A, 5
Fonseca, RJC, 1
Fontenelle, M, 20

Francis, D, 27
Freitas, VM, 36
Gamero, EJP, 10
Gantus, MAV, 10
Gastardelo, TS, 33
Georgii, J, 43
Gil, CD, 15
Gil, LM, 13
Godoy, JAP, 40
Gomes, AM, 1
Gomes, JR., 5
Gomes, RCT, 48
Hetzl, A, 5
Higuchi, DA, 42
Ignacio, KL, 22
Justulin Jr, LA, 26
Kühne, F, 11
Lacorte, LM, 38
Laura, ALC, 53, 53
Leão, ACM, 31
Lima, MMC, 29
Lima, MT, 37
Lima, R, 9
Machado, CF, 30
Machado, D, 23
Machado, DE, 24
Maciel, TSF, 14
Marangoni, RG, 18
Marques, MF, 39
Martins, V, 37
Massaro, RR, 35
Matamoros, SEB, 10
Medeiros Junior, JL, 49
Medeiros, VP, 3, 40
Melo, AV, 19
Melo, FR, 11
Mendez, CH, 11
Mendonça, L, 42
Menezes, K, 42
Meuser-Batista, M, 17
Monteiro, JC, 8
Morais, J, 10
Nakagaki, WR, 7

Nascimento, AP, 45
Nascimento, CF, 34
Nascimento, FD, 36
Nascimento, MA, 18
Nascimento, S, 32
Nesi, RT, 16
Nones, J, 29
Oliveira, AL, 48
Oliveira, LZ, 32
Omar, NF, 5
Pádua, T, 43
Pelli, AA, 2
Pereira, EGJ, 17
Pereira, RMC, 14
Perez, APS, 27
Petito, RB, 45, 47
Petrópolis, D, 21, 21
Peyerl, E, 13
Pinheiro, JJV, 36
Pinto, FCM, 47
Pitanga, BPS, 39
Polli, VAB, 13
Portilho, D, 12
Possidonio, ACB, 12
Prieto, T, 54
Ramos, TDP, 19
Ribeiro, G, 44
Ribeiro, LM, 49
Ribeiro, MNP, 6, 7
Ribeiro, RF, 52
Riederer, I, 17, 42
Rocha, C, 16
Rocha, VN, 46, 46
Rochel, SS, 49
Rodart, IF, 3
Sá, VK, 38

Salgado, RM, 52
Sanches, J, 51
Sant'Anna, MMS, 15
Santos, BL, 39
Santos, ES, 33
Santos, FCA, 43
Santos, JS, 51
Santos, P, 1
Santos, SAA, 8
Santos, TS, 51
Sarobo, C, 26
Scarano, WR, 50
Silva, CMC, 52
Silva, CS, 1
Silva, FS, 6
Silva, TA, 20
Silva, VA, 51
Siqueira, AS, 35, 48
Sousa, GLSC, 8
Souza, BR., 50
Souza, CS, 30
Souza, LNG, 40
Souza, PC, 33
Spinetti, E, 41
Spohr, TCL, 27
Stipursky, J, 28
Taboga, SR, 24
Teixeira, J, 44
Teixeira, LAC, 2
Theodoro, TR., 31
Tomiosso, TC, 9
Torres, PHM, 38
Velosa, APP, 18
Vilanova, E, 4

INDEX

A

- absorption, 14
- access, 169, 170, 276, 307
- accessibility, 327
- acclimatization, 128, 130
- accounting, 93
- accuracy, 153, 169
- acetate, 182
- acetone, 79, 195, 197
- acetonitrile, 195
- acid, 46, 142, 159, 176, 196, 197, 198, 199, 201, 203, 230, 242, 246, 259, 263, 270, 288, 289, 292, 293, 296, 297, 298, 306, 318, 341, 362, 397, 416
- acrosome, 284
- ACTH, 139
- activation, 167
- active site, 389
- acute, 179
- adaptation, 278, 429
- adaptations, 12, 278
- adductor, 38
- adenocarcinoma, 389, 393
- adhesion, x, xi, 36, 37, 38, 39, 40, 41, 42, 45, 54, 55, 210, 216, 217, 246, 273, 277, 278, 280, 281, 282, 292, 299, 312, 320, 321, 323, 327, 330, 332, 339, 340, 344, 345, 373, 377, 380, 384, 385, 387, 388, 389, 394, 395, 401, 402, 435
- adhesions, 384
- adhesive properties, 311
- adhesives, 279
- adipose, 287, 336, 356, 400, 402, 403
- adipose tissue, 287, 336, 356, 400, 402
- adjustment, 187, 191
- administration, 162, 163
- ADP, 311
- adult, xiii, 127, 128, 129, 158
- adulthood, ix, 17, 18, 23, 24, 72, 424
- adults, 129
- adventitia, 286
- Africa, 118, 124, 154, 206
- agar, 129
- age, 3, 4, 14, 71, 72, 73, 74, 115, 153, 165, 190, 234, 238, 300, 308, 309, 327, 328, 339, 360, 372, 422
- agencies, 202
- agent, 168, 174
- agents, 133, 134, 137, 159, 167, 169
- aggregates, 282, 299
- aggregation, xi, 28, 30, 38, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 279, 282, 299, 349, 389, 395
- aggressiveness, 348
- agonist, 311
- agricultural, 174
- agricultural sector, 38
- agriculture, 174
- air, 133, 169
- air-dried, 133
- airways, 354
- alanine, 296
- alanine aminotransferase, 296
- albumin, 263, 335
- alcohol, 168, 169, 410
- alcohol consumption, 410
- algae, 109, 295
- alkalinity, 128
- allele, 251
- allergy, 331
- alopecia, 207
- alternative, 153
- alters, 267, 345, 360, 409
- alveolar macrophage, 183
- alveolus, 361
- amines, 167
- amino, 158, 162, 163
- amino acid, 162
- amino acids, 162, 392, 421
- amphibia, 293, 312

- amphibians, 294
 amplitude, 3, 7, 88
 amylase, xv, 193, 197, 201
 anaesthesia, 132
 anatomy, 2, 13, 256, 415
 anchoring, 364
 androgen, 301, 309, 423
 androgens, 306, 361, 426
 angiogenesis, 227, 280, 281, 305, 306, 319, 348, 356, 358, 374, 377, 379, 393, 394, 396, 397, 425
 angiogenic process, 402
 angioplasty, 398
 angiotensin II, 349
 animal pole, 259
 animal welfare, 84
 animals, xiv, 151, 153, 158, 161, 162, 173, 174, 175, 176, 179, 182
 Animals, 174
 annotation, 257
 antagonism, 235
 antagonists, 162
 antibiotic, 302, 375
 antibody, 139, 159, 207, 211, 212, 215, 236, 256, 261, 277, 319, 332, 334, 337, 367, 374, 404, 415, 430
 anti-cancer, 306
 anticoagulant, 30, 132, 274, 284, 287, 289, 290, 291, 292, 293, 294, 295, 315, 347
 antigen, 212, 219, 331, 350, 353, 425
 antigenicity, 336
 anti-inflammatory drugs, 412
 antinuclear antibodies, 331, 337
 antioxidant, 202, 426, 428
 antipsychotic, 235
 antipsychotic drugs, 235
 antisense, 236
 antitumor, 295, 371, 391
 aorta, 292, 295, 357, 409
 APC, 390
 apnea, 355
 apoptosis, 167, 218, 236, 241, 246, 282, 311, 329, 330, 335, 338, 339, 340, 353, 371, 377, 378, 385, 389, 391, 423, 435
 appetite, 207
 applications, 275, 288, 321, 431
 aquatic systems, 236
 Arabia, 151
 Arabian Peninsula, 186
 Arabidopsis thaliana, 236, 268, 269
 Argentina, 67, 227, 273, 282
 arginine, 174, 305, 313, 421
 argon, 347
 aromatic hydrocarbons, 167
 arrest, 47, 340, 367, 392
 arsenic, x, xi, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 43, 45, 46, 47, 49, 52, 53, 174, 176, 179, 180, 181, 182, 183
 arsenite, xiv, 173, 174, 176, 177, 178, 179, 183
 arterial blood gas, 410
 arteries, 282, 284, 289, 409, 431
 arterioles, 409
 artery, 286, 349, 398, 410
 arthritis, 394
 arthroplasty, 401
 arthropods, 55
 articulation, 336, 417
 ascites, 170
 ash, 4, 8
 ASI, 420
 Asia, 150, 206
 aspartate, 158, 159
 aspiration, 168
 assessment, 13, 82, 101, 128, 138, 182
 assessment tools, 13
 assignment, 203
 asthma, 330, 331
 astrocytes, 362, 364, 365, 366, 368
 asymmetric synthesis, 263
 asymptomatic, xv, 131, 205, 207, 211, 212, 213, 214, 216, 217, 219, 220, 296, 353
 atherogenesis, 286
 atherosclerosis, 286, 292, 293, 319
 Atlantic, 139
 Atlas, 171
 atoms, 392
 ATP, 78, 311
 atria, 263
 atrophy, 332, 353, 413
 attachment, 52, 53, 311, 327
 Australia, 115, 238
 authentication, 152
 authors, xvi, 42, 71, 72, 83, 84, 94, 101, 138, 186, 198, 202, 208, 209, 211, 235, 305, 307, 317, 398
 autoantibodies, 331, 332, 336
 autoimmune disease, 331, 335, 336, 340
 autoimmunity, 316, 333, 336, 337
 autopsy, 158
 availability, 9, 29, 186, 290, 320, 357
 averaging, 19, 22, 24
 axonal degeneration, 402
 axons, 236, 248, 266

B

- Bacillus subtilis, xv, 193, 197, 201
 back pain, 291

- background, 170, 241, 276, 382
bacteria, 129, 190
bacterial, xiii, 127, 129, 130, 138, 139
bacterial infection, 138, 211
bacterial strains, 129
Bahrain, 186
Bangladesh, 35, 54, 174, 183
barriers, 280, 283, 382
basal lamina, 251, 312, 414, 432
base pair, 120
basement membrane, 264, 265, 280, 281, 309, 312, 314, 347, 352, 375, 380, 383, 387, 396, 413, 430, 431, 432
basophils, 133
BBB, 288, 372, 402
behavior, xii, 18, 23, 96, 99, 151, 186, 187, 190, 207, 238, 269, 272, 273, 276, 278, 281, 317, 322, 326, 345, 358, 372, 377, 383, 384, 387, 392, 403, 409, 432
Belgium, 60
bending, 262, 308
beneficial effect, 402
benign, 85, 166, 167, 170, 301, 379, 408, 426
benign prostatic hyperplasia, 301, 408
Beta-adrenoceptor, 163
bile, 400
bile duct, 400
biliary atresia, 400
binding, xii, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 159, 163, 208, 218, 220, 227, 228, 230, 238, 239, 242, 255, 260, 274, 276, 277, 279, 282, 283, 284, 290, 295, 315, 329, 330, 339, 346, 352, 370, 376, 379, 380, 397
bioassay, 129
bioavailability, 194, 294
biochemistry, 300
biocompatibility, 322, 323
biodiversity, xi, 46, 182, 186
bioinformatics, 230, 242, 248, 260, 268
biological activity, 274, 284, 295, 396
biological control, xv, 185, 186, 190
biological processes, 230, 249, 332, 339, 362
biological responses, 281, 362
biomarker, 128, 173, 179, 182, 183
biomarkers, xiv, 139, 173, 174, 182, 183, 428
biomass, ix, 1, 4, 8, 11, 13
biomaterials, 322, 350
biomechanics, 313
biopolymer, 289
biopsies, 165
biopsy, 74, 166, 296, 419, 427
biosphere, x, xi, 27, 28, 45, 46
biosynthesis, 64, 259, 261, 283, 292, 311, 313, 352
biotechnology, 311
biotin, 69, 295, 329
birds, 153, 154
birefringence, 310, 411
birth, 241, 308, 328, 329, 357, 359, 417, 429
bivalve, 173, 179, 182, 183
bladder, 305, 306, 307, 359, 407
blastocoele, 228
blastula, 228, 254
bleaching, 194
bleeding, 38, 169, 170, 207, 282, 289, 294, 347, 349, 355, 402
bleeding time, 349
blocks, 318, 333
blood, x, xi, xiii, xiv, xv, 28, 30, 35, 36, 37, 38, 42, 45, 46, 47, 52, 53, 54, 127, 128, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 153, 157, 158, 159, 160, 161, 162, 174, 175, 205, 207, 211, 212, 216, 220, 231, 232, 282, 284, 286, 290, 296, 312, 315, 328, 329, 330, 352, 353, 355, 356, 394, 400, 402, 405, 406, 407, 409, 410, 414, 415, 423, 425, 426, 431, 432, 434
blood flow, 414, 415, 431
blood glucose, xiv, 157, 159, 160, 162
blood monocytes, 220
blood plasma, 423
blood pressure, 284, 315, 409, 410, 414
blood smear, 133
blood stream, 35
blood supply, 352
blood vessels, xv, 205, 207, 312, 352, 402, 405, 406, 407, 423, 425
bloodstream, 344, 380
body fluid, xi, 45, 54
body size, ix, xii, 1, 11, 99, 248
body weight, xiv, 78, 129, 157, 158, 163, 186, 305, 306, 307, 309, 407, 410, 416, 430, 434
bolus, 153
bonds, 310
bone, xv, 79, 84, 85, 86, 205, 206, 207, 208, 210, 212, 250, 298, 302, 308, 309, 321, 322, 323, 324, 326, 327, 329, 336, 350, 361, 398, 400, 403
bone form, 350, 362
bone marrow, xv, 205, 206, 207, 208, 210, 212, 329, 398, 400, 403
bone mass, 308
bones, 250, 344
borderline, 162
bowel, 72, 294, 355
bowel obstruction, 355
bradykinin, 55
brain, 248, 266, 288, 362, 364, 365, 366, 372, 394, 395

brain stem, 288
 brain structure, 248
 brain tumor, 395
 branching, 241, 274, 283, 358, 359, 367
 Brazil, v, vi, ix, xii, 1, 2, 8, 10, 13, 14, 79, 84, 87, 88, 89, 91, 92, 93, 95, 96, 97, 165, 167, 193, 205, 206, 210, 215, 219, 228, 235, 238, 239, 240, 243, 244, 252, 253, 255, 260, 261, 266, 267, 272, 273, 274, 275, 277, 278, 279, 280, 281, 282, 283, 284, 286, 287, 288, 289, 310, 313, 329, 338, 343, 347, 357, 359, 371, 378, 379, 389, 391, 392, 394, 395, 401, 408, 413, 422, 425, 427
 Brazilian, xiv, 165, 170
 breakdown, 52, 309, 357, 383
 breast cancer, 374, 376, 377, 389, 394
 breast carcinoma, 388
 breeding, xiv, 9, 174, 185, 186, 187, 189, 190
 bronchioles, 333
 bronchitis, 432, 433
 browser, 233
 buffer, 30, 59, 79, 290, 297, 302, 330, 338, 347, 353, 361, 387, 391
 Burma, 115
 bursa, 114

C

C reactive protein, 287, 338
 cabbage, 18
 cadmium, 391
 caecum, 72
 calcium, 3, 163, 243, 244, 253, 299, 369, 370, 391
 calcium carbonate, 3
 caliber, 431
 calorimetry, 163
 calvaria, 350
 calyx, 248
 Canada, 14, 239, 287, 369
 canals, 186
 cancer, xiv, 165, 166, 167, 168, 169, 170, 271, 273, 276, 281, 282, 301, 305, 306, 374, 375, 376, 377, 378, 379, 380, 383, 385, 386, 388, 389, 390, 391, 393, 394, 397, 414, 421, 423
 cancer cells, 380
 cancer progression, 276, 388
 cancerous cells, 170
 candidates, 259, 264, 327, 381
 capillary, 59, 133, 196, 347, 352, 367, 394
 capsule, ix, 1, 5, 100, 410
 carapace, 11, 29, 47
 carbohydrate, xi, 67, 68, 73, 274, 282, 293, 299, 379, 380
 carbohydrates, 49, 58, 203

carbon, 12, 199, 203, 321
 carbon nanotubes, 321
 carcinogenesis, 168, 375, 378, 379, 391
 carcinogenic, 167, 168
 carcinoma, 165, 168, 273, 281, 374, 375, 378, 382, 383, 384, 386, 387, 388, 396, 397
 cardiac function, 161
 cardiac muscle, xiv, 157, 158, 161, 328, 342
 cardiomyopathy, 335, 341
 cardiovascular disease, 410, 422
 cardiovascular risk, 409
 cardiovascular system, 284, 360
 carotene, 194
 carotenoids, 194
 carrier, 131
 cartilage, 84, 86, 245, 250, 323, 324, 326, 327, 336, 356, 399, 403
 cartilaginous, 327, 361
 case study, 190
 caspases, 378
 cast, 209
 castration, 358, 413, 414, 423
 catecholamines, 410
 catfish, 118, 124
 cation, 208, 346
 cavities, 170
 cell culture, 78, 271, 277, 288, 318, 325, 326, 347, 363
 cell cycle, 167, 243, 246, 282, 304, 320, 377, 385, 391
 cell death, 218, 224, 230, 231, 249, 266, 311, 335, 366, 377, 389, 391, 404, 435
 cell division, 167, 170
 cell fate, 363, 364
 cell fusion, 320
 cell growth, 167
 cell invasion, 280, 383, 430
 cell line, 74, 86, 213, 219, 227, 235, 288, 319, 370, 371, 374, 375, 376, 377, 381, 382, 383, 384, 385, 386, 388, 389, 394, 396, 397, 401, 411
 cell lines, 86, 219, 235, 288, 371, 376, 377, 381, 386, 389, 394, 397, 401
 cell membranes, 321
 cell signaling, 228, 254, 277, 347, 358
 cell surface, 77, 79, 80, 211, 277, 282, 287, 306, 330, 332, 339, 340, 351, 369, 372, 373, 379, 386
 cellular atypia, 170
 cellular homeostasis, 278
 cellular immunity, 216, 285
 cellulose, 290
 central nervous system, 237, 287, 317, 363
 centriole, 79, 80, 81, 83
 cerebellar development, 368

- cerebellum, 367, 369, 373
cerebral cortex, 363, 364, 365, 366, 367
cerebrovascular disease, 282
cervical cancer, xiv, 165, 166, 167, 169, 170
cervical dysplasia, 168
cervical screening, 169
cervix, 165, 166, 417
challenges, 202
channels, 261
character, 101, 114, 252, 395, 402
charge density, 289
chelicera, 147, 149
chemiluminescence, 138
chemokine receptor, 215, 219, 221, 340
chemokines, 211, 276, 334
chemotaxis, 383, 388, 394
chemotherapy, 166, 215, 375, 394
chicken, 233, 237, 242, 250, 263, 325
children, 206
China, 150
chloroform, 158
chlorophyll, xii, 87, 90, 91, 94
cholesterol, 228, 254, 259, 320, 381
chondrocyte, 361
choriocarcinoma, 351
chromatography, 197, 289, 290, 291, 293, 298
chromosome, 152, 167, 252, 374, 376
chromosomes, 167
chronic renal failure, 410
cigarette smoking, 183
cilia, xii, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86
cilium, xii, 77, 80, 81, 83, 85, 86
CIN, 165, 168, 170
circulation, xii, 87, 380
cirrhosis, 400
classes, 4, 8, 167
classification, xiii, 36, 42, 55, 99, 101, 111, 115, 118, 169, 170, 349
cleaning, 101, 266
cleavage, 58, 243, 339, 376, 383, 387, 389, 396, 411
cleavages, 374
climate, 2, 88
clinical examination, 169
clinical symptoms, 212, 338, 408
clone, 234, 386
cloning, 65
closure, 55, 246, 299, 318, 395, 425
cluster analysis, 90
clusters, 248, 257, 264, 317
CMC, 439
CNS, 249, 317, 363, 372, 373
oagulation, 284, 286, 289, 290, 349
coagulation factors, 284
cobalt, 391
cocoon, 263
coding, 237, 370
codon, 370
coffee, 106, 109, 112, 272, 273, 274, 275
cohort, 4, 207, 217, 220
collage, 322
collagen, 84, 279, 285, 297, 298, 299, 300, 303, 306, 307, 310, 313, 314, 315, 316, 319, 321, 322, 323, 324, 325, 326, 327, 331, 333, 335, 336, 337, 345, 346, 352, 354, 356, 357, 358, 359, 361, 367, 375, 378, 387, 389, 391, 392, 395, 400, 401, 402, 405, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 422, 423, 424, 425, 427, 428, 429, 431, 432, 433, 434
colon, xii, 67, 68, 70, 71, 72, 294, 306
colonization, 68, 105
color, iv, xiii, 100, 194, 197, 201, 421
colorectal cancer, 381
colposcopy, 165, 170
communication, 52, 97, 141, 271, 273
community, xii, 12, 14, 87, 88, 90, 93, 97
competition, 401
complement, 267, 339
complexity, 9, 290, 312, 335, 362, 366
compliance, 354
complications, 305, 306, 309, 354, 409, 430
components, 13, 82, 84, 166, 167, 202, 228, 230, 231, 241, 255, 264, 265, 267, 278, 279, 284, 285, 288, 290, 296, 297, 313, 314, 317, 327, 335, 342, 345, 346, 352, 354, 356, 357, 361, 365, 372, 375, 391, 394, 396, 400, 401, 404, 415
composition, ix, xv, 71, 72, 73, 88, 90, 95, 128, 139, 193, 194, 254, 284, 298, 327, 355, 433
compound eye, 100, 251, 255
compounds, 53, 153, 269, 277, 290, 317, 366, 371, 395, 418
compression, 84, 287, 310, 371, 402
concentration, x, 29, 30, 35, 37, 39, 46, 47, 49, 52, 53, 61, 69, 95, 129, 130, 153, 163, 174, 175, 179, 186, 194, 197, 228, 239, 243, 244, 254, 262, 285, 291, 292, 297, 298, 304, 317, 319, 341, 352, 391, 405, 406, 410, 432
condensation, 265, 360
conformity, 179
connective tissue, 82, 84, 85, 210, 211, 217, 294, 312, 323, 325, 350, 354, 389, 425, 434
consensus, 119, 122, 123
conservation, xv, 25, 124, 151, 154, 185, 186, 190
constant rate, 207
construction, 411
consumers, 28, 46

consumption, 35, 191, 202, 307, 409, 410, 430, 432, 433
 contaminant, 174, 290
 contamination, xi, 28, 29, 45, 46, 47, 174, 182, 288
 control condition, 371, 395
 control group, 78, 130, 136, 158, 159, 160, 210, 305, 306, 307, 308, 338, 353, 354, 356, 360, 361, 392, 400, 405, 406, 407, 408, 410, 413, 421, 422, 425, 428, 429, 430, 432, 433
 control measures, 207
 conversion, 163, 174, 324
 copper, 36, 43, 202
 copulation, 21, 23, 24
 corn, 423, 424
 correlation, 3, 5, 6, 58, 209, 213, 254, 258, 259, 270, 291, 296, 301, 330, 334, 345, 376, 381, 384, 390, 418
 correlation analysis, 3
 correlation coefficient, 5
 correlations, 9, 211, 386
 corrosion, 322
 cortex, 257, 288, 339, 363, 364, 365, 366, 367, 371
 cortical bone, 308
 cortical neurons, 362, 363
 cost, 169, 186
 Costa Rica, 12
 cost-effective, 169
 coupling, 199
 covering, 107, 350
 creatine, 434
 credibility, 182
 Creutzfeldt-Jakob disease, 370
 critical period, 307
 crops, 17
 cross-sectional study, 218
 crust, 174
 crystalline, xv, 58, 193, 197, 201
 crystals, 137
 cues, 278, 372
 cultivation, 345, 346
 culture, 39, 48, 129, 137, 173, 194, 211, 241, 263, 265, 271, 277, 288, 292, 299, 300, 302, 314, 316, 318, 319, 320, 323, 324, 325, 327, 332, 343, 348, 358, 363, 365, 366, 367, 374, 375, 397
 culture conditions, 263
 culture media, 288, 397
 cuticle, 100, 258, 267
 cycles, 59, 60, 195, 198, 308, 406
 cyclooxygenase, 412, 429
 cyst, 264, 348
 cytochrome, 118, 124, 152, 435
 cytocompatibility, 322

cytokines, 73, 209, 211, 212, 213, 214, 285, 330, 335, 342
 cytokinesis, 230
 cytology, xiv, 165, 166, 169, 170
 cytometry, 210, 277, 304, 329, 330, 370, 386, 391, 403, 411, 416
 cytoplasm, 166, 170, 231, 255, 261, 293, 304, 354
 cytosine, 307
 cytoskeleton, 230, 246, 249, 262, 311, 320, 342, 343, 384
 Cytotoxic, 173
 cytotoxicity, 182, 347, 375, 391

D

damages, iv, 236
 Darjeeling, 173
 data analysis, xiii, 117, 176, 317
 data distribution, 90
 data gathering, 392
 data set, 119, 121
 data structure, xiii, 117
 database, 259, 263, 268
 DBP, 347, 424
 death, ix, 17, 18, 22, 23, 24, 53, 166, 167, 218, 224, 230, 231, 249, 266, 311, 332, 335, 366, 377, 378, 389, 391, 400, 404, 426, 435
 death rate, 379
 deaths, 374
 decomposition, xii, 2, 13, 99
 defects, 228, 246, 265, 318, 350
 defence, 55
 defense, xii, 42, 75, 99, 183, 235, 240, 282, 285
 defenses, 167
 deficiencies, 267, 318
 deficiency, 13, 170, 212, 242, 276, 286, 311, 340
 degradation, 152, 162, 186, 201, 253, 267, 277, 310, 348, 352, 357, 375, 382, 384, 386, 388, 391, 412, 424, 431
 delivery, 304, 417, 430, 435
 demyelination, 338
 dendrites, 248
 dendritic cell, 215, 221
 density, x, xii, 5, 6, 8, 9, 27, 30, 35, 47, 87, 93, 94, 183, 217, 220, 247, 254, 289, 295, 305, 306, 343, 345, 353, 368, 379, 380, 405, 412, 425, 429
 density values, 94
 dental implants, 322
 deposition, 280, 284, 285, 299, 309, 313, 319, 332, 333, 334, 336, 356, 359, 364, 404, 408, 413, 416, 417, 431, 433, 434
 deposits, 413
 depressed, xiii, 127

- depression, x, xiii, 27, 103, 105, 127, 137
 deprivation, xiv, 157, 309
 deregulation, 158
 derivatives, 100, 196, 245, 288, 323, 325, 326
 dermatitis, 207
 dermis, 209, 293, 299, 316, 332, 375
 desiccation, 10, 29
 destruction, 137, 336, 340, 402
 detachment, 367
 detection, xiv, 59, 165, 166, 169, 170, 195, 197, 216, 270, 279, 298, 406, 422, 429
 developed countries, 291
 developing brain, 364
 developing countries, 166, 375
 developmental process, 233, 235, 257
 diabetes, 162, 309, 340, 356, 429, 430
 diabetic patients, 356
 dialysis, 197, 199
 diaphysis, 308
 diarrhea, 207
 diet, 52, 59, 73, 154, 158, 262, 286, 305, 306, 307, 360, 410, 412, 413, 422
 dietary, 153, 173
 diets, 150
 differentiation, 68, 73, 74, 85, 166, 167, 225, 230, 231, 232, 237, 241, 248, 249, 255, 257, 258, 264, 265, 270, 271, 274, 275, 281, 283, 284, 289, 311, 312, 317, 318, 319, 320, 321, 323, 324, 325, 327, 329, 332, 340, 359, 361, 362, 363, 364, 365, 366, 371, 385, 386, 387, 388, 389, 393, 394, 397, 398, 399, 400, 404, 425, 429
 diffusion, 283
 digestibility, xv, 193
 digestion, 43, 59, 382, 389, 399
 dilation, 286
 dimethylsulfoxide, xv, 193
 dimorphism, 238
 direct action, 301
 directionality, 265
 disability, 348
 discontinuity, 410
 discrimination, 41
 discs, 255, 256, 291
 disease model, 335, 336
 disease progression, 206, 301
 diseases, 137, 138, 140, 167, 168, 169, 170, 272, 274, 276, 282, 287, 288, 290, 299, 300, 304, 330, 340, 367, 369, 370, 391, 401, 410, 426
 disintegrin, 352, 388, 401, 402
 dislocation, 301, 302
 disorder, 361, 414
 dispersion, 323
 displacement, 411
 disposition, 150, 168, 421
 disseminate, 207, 208, 344
 dissolved oxygen, xii, 87, 91, 94, 128, 194
 distilled water, 47, 69, 194, 195, 197, 420
 distribution, ix, xiii, 4, 6, 9, 10, 13, 14, 32, 39, 72, 96, 100, 162, 210, 229, 254, 272, 279, 293, 294, 303, 316, 321, 328, 329, 335, 342, 352, 353, 355, 358, 359, 379, 413, 417, 422, 427, 430, 433
 disturbances, 28, 46, 128, 264
 divergence, 120, 121, 257
 diversity, xii, 28, 46, 87, 90, 91, 93, 94, 95, 115, 118, 124, 154
 division, 166, 167, 170, 224, 225, 232, 243, 259, 304
 DNA, vi, ix, xiv, 59, 60, 61, 64, 66, 124, 125, 151, 152, 153, 154, 155, 167, 168, 221, 227, 234, 245, 252, 270
 DNA extraction, 153
 DNA polymerase, 60, 234
 DNase, 59
 dogs, xv, 78, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221
 donors, 296, 353
 dopamine, 235
 dosage, 297, 348, 420, 432
 down-regulation, 328
 drainage, 191
 drinking water, 313
Drosophila, 58, 65, 224, 227, 228, 230, 239, 241, 245, 249, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 263, 266, 267
 DRS, 54, 182
 drugs, 235, 277, 285, 290, 294, 295, 321, 371, 375, 392, 412, 413, 421
 drying, 168, 186, 197, 416
 D-serine, 363
 duration, 23, 38, 283, 310, 409, 430
 Duration, 19, 20, 21
 duties, 266
 dynamic loads, 327
 dynamics, 2, 11, 12, 13, 14, 15, 25, 154, 216, 231, 241, 252, 269, 276, 311, 358, 392
 dysmenorrhea, 352, 355
 dyspareunia, 355
 dysplasia, 166, 168
 dysuria, 306

E

- ear cyst, 348
 early retirement, 291
 ears, 182
 earth, 174

- ECM, 241, 271, 272, 273, 274, 275, 277, 279, 280, 281, 283, 284, 285, 289, 295, 297, 299, 300, 309, 310, 311, 313, 314, 319, 320, 323, 324, 325, 328, 334, 339, 341, 342, 343, 344, 345, 346, 347, 348, 353, 357, 361, 362, 364, 368, 371, 373, 374, 375, 379, 382, 386, 387, 388, 395, 398, 401, 404, 409, 411, 416, 422, 430, 433
- ECM degradation, 382, 386, 388
- ecology, 14, 101, 104, 106, 109, 111, 182, 191
- ecosystem, x, xi, 27, 28, 29, 35, 38, 45, 46, 52, 54, 182
- ecotoxicology, 139
- ectoderm, 245, 262
- edema, 430
- effluent, 139
- effluents, 138
- egg, ix, xiv, 4, 5, 6, 9, 10, 12, 17, 22, 23, 25, 64, 185, 187, 189, 190, 243, 244, 246, 257, 283
- Egypt, ix, 17, 18, 57, 186, 191
- elasticity, 310, 418
- elastin, 284, 346, 359, 407, 409, 415, 418, 431
- elderly, 162, 301
- electrolyte, 293
- electron, 79, 80, 85, 111, 169, 228, 251, 264, 270, 277, 292, 322, 326, 330, 345, 354, 413, 414, 415, 418, 430
- electron microscopy, 228, 264, 270, 277, 292, 322, 330, 345, 354, 413, 414, 415, 418, 430
- electrophoresis, 234, 291, 342, 375, 376
- electroporation, 242, 262
- elongation, 84, 247, 368
- elucidation, 203
- e-mail, 157, 251
- embryo, 58, 224, 228, 229, 236, 237, 239, 247, 250, 251, 253, 254, 256, 264, 268, 278, 280, 306, 324, 326, 342, 430
- embryogenesis, 228, 237, 243, 256, 267, 268, 361, 426
- embryonic stem cells, 275
- emission, 308, 345, 353
- encephalitis, 346
- encoding, 225, 258
- endangered species, 118, 124
- endocardium, 328
- endocrine, 158, 162, 245, 307, 318, 325, 326, 397, 426
- endocrine-disrupting chemicals, 426
- endoderm, 245, 251
- endometriosis, 352, 355
- endothelial cells, 272, 276, 277, 278, 291, 295, 312, 319, 337, 341, 347, 380, 394, 398
- endothelial dysfunction, 337, 398
- endothelium, 276, 286, 315, 333, 336, 337, 380, 398, 414, 415
- endurance, 310
- energy, xii, 10, 12, 99, 284, 307, 315, 339, 392
- energy transfer, 315, 339
- engineering, 65, 399, 415, 431
- environment, x, 10, 12, 14, 27, 28, 29, 30, 35, 38, 53, 84, 128, 139, 174, 186, 208, 240, 250, 292, 322, 323, 345, 375, 425
- environmental change, 194, 285
- environmental conditions, 152, 153, 186, 207
- environmental contamination, 53, 182
- environmental factors, 29, 88, 186, 190, 191
- enzymatic activity, 339, 361, 374, 397
- enzyme-linked immunosorbent assay, 207
- enzymes, 54, 59, 60, 61, 139, 140, 241, 258, 277, 283, 301, 348, 354, 357, 360, 382, 383, 388, 391, 394, 396, 397, 407, 422, 423
- eosinophilia, 137
- eosinophils, 133
- epidemic, 412
- epidemiologic studies, 206
- epidemiology, 209, 219, 354
- epidermis, 159, 162, 258, 312, 325, 326, 429
- epithelia, 86, 166, 229, 312, 387, 423
- epithelial cell, 153, 166, 170
- epithelial cells, 68, 77, 83, 153, 166, 170, 277, 307, 312, 332, 339, 347, 386, 423, 426
- epithelium, 65, 68, 70, 73, 74, 84, 85, 229, 241, 264, 265, 301, 303, 307, 323, 358, 360, 407, 408, 416, 424, 427
- Epstein-Barr virus, 167
- equipment, 168, 327
- erosion, 348
- erythrocyte, 137
- erythrocyte membranes, 339
- erythrocytes, 338, 341
- esophagus, 337
- EST, 233, 255, 256, 268
- ester, 313
- estimating, 47, 84, 139
- estrogen, 303, 306, 307, 314, 315, 361, 420, 426, 430, 431
- estuarine systems, 94
- ETA, 334
- ethanol, 59, 69, 101, 195, 197, 269, 348, 432
- ethics, 54, 293, 312
- ethyl acetate, 101, 197
- ethyl alcohol, 168
- ethylene, 46
- ethylenediamine, 176
- etiology, 316
- europium, 295

euthanasia, 412, 414, 420, 425, 429
 evaporation, 195
 evolution, 78, 120, 124, 166, 219, 247, 255, 269,
 280, 283, 289, 298, 336, 339, 434
 excitation, 353
 exclusion, 38, 47, 175, 197, 375
 exercise, 298, 310, 409
 exocytosis, 83, 284
 exoskeleton, 258
 experimental condition, 82
 exploitation, 186
 exposure, x, xi, xiii, 11, 27, 28, 31, 34, 35, 36, 37,
 38, 39, 45, 46, 47, 48, 49, 50, 52, 53, 55, 69, 127,
 128, 130, 131, 133, 134, 135, 136, 137, 168, 174,
 176, 177, 178, 179, 212, 227, 288, 291, 307, 371,
 375, 394, 395, 424, 426, 431, 435
 Exposure, 173
 expressed sequence tag, 268
 extinction, 186
 extracellular matrix, 82, 210, 211, 241, 246, 272,
 275, 277, 278, 279, 280, 282, 283, 284, 285, 287,
 288, 294, 295, 296, 297, 299, 301, 303, 306, 309,
 310, 312, 313, 314, 315, 317, 319, 320, 322, 323,
 325, 327, 330, 332, 335, 339, 340, 345, 346, 347,
 348, 349, 351, 353, 354, 356, 357, 359, 362, 363,
 364, 368, 372, 374, 375, 376, 380, 382, 383, 386,
 387, 388, 389, 391, 393, 394, 396, 397, 400, 401,
 404, 407, 410, 411, 413, 414, 416, 423, 429, 430,
 431, 433, 434, 435
 extraction, xv, 30, 47, 142, 153, 193, 195, 198, 270,
 299, 302, 348, 377, 403, 420, 424
 extravasation, 380

F

fabrication, 322
 faecal, 154
 failure, 120, 161, 163, 246, 276, 338, 400
 family, 2, 114, 115, 124, 227, 230, 235, 238, 250,
 255, 256, 259, 261, 263, 268, 269, 282, 283, 295,
 309, 319, 330, 332, 339, 340, 351, 372, 373, 379,
 388, 390, 391, 394, 430
 family members, 390
 fantasy, 154
 fasting, 159
 fat, xi, 57, 58, 59, 61, 62, 63, 64, 65, 230, 238, 240,
 261, 286, 412, 413
 fauna, 9, 14, 15, 97, 115
 fear, 170
 feces, xiv, 151, 153, 154
 feeding, 150, 173
 feet, 112

females, ix, xiii, xiv, 1, 4, 5, 59, 64, 65, 99, 101, 102,
 114, 152, 185, 187, 190, 230, 238, 253, 266, 296,
 316, 360, 422, 424, 426, 429
 femur, 142, 308
 fertility, 252, 355
 fertilization, 235, 243, 244, 274, 283
 fetal development, 284, 422
 fetal growth, 360
 fetus, 351, 432
 fever, 207
 fiber, 284, 300, 314, 320, 345, 349, 354, 358, 399,
 409, 414, 415, 418, 434
 fibers, 83, 84, 285, 288, 299, 300, 303, 313, 314,
 316, 326, 327, 336, 352, 354, 355, 356, 358, 359,
 361, 384, 389, 402, 405, 406, 407, 408, 409, 410,
 412, 414, 415, 417, 421, 422, 424, 425, 429, 431
 fibrinogen, 282, 349
 fibroblast proliferation, 378
 fibroblasts, xii, 77, 78, 79, 81, 82, 83, 84, 85, 86,
 245, 279, 288, 299, 300, 313, 314, 316, 325, 328,
 333, 337, 342, 359, 378, 389, 401, 419, 423, 426,
 427, 435
 fibrosis, 274, 285, 286, 296, 314, 316, 328, 331, 333,
 335, 337, 342, 355, 400, 410, 416, 422, 423, 432
 fibrous tissue, 297, 356, 399
 field crops, 18
 films, 133
 filtration, 289, 291
 financial support, 11, 42, 54, 84
 fingerprinting, 153
 fish, xiii, xiv, 12, 14, 88, 95, 127, 128, 129, 130, 131,
 132, 134, 135, 136, 137, 138, 139, 140, 185, 186,
 187, 189, 190, 194, 259
 fish disease, 138, 140
 fission, 202, 203
 fitness, 182, 262
 fixation, 30, 68, 163, 168, 169, 382, 413
 FL, 183
 flavonoids, 366, 371, 394, 395, 396
 flood, 88, 90, 94, 95
 flora, 28, 46, 75
 flora and fauna, 28, 46
 fluid, 84, 86, 133, 170, 330
 fluorescence, 304, 317, 339, 372, 382
 fluorescent light, 169
 FMC, 342
 focusing, 256, 346, 360
 folic acid, 246
 follicle, 211, 257, 327
 follicles, 160, 162, 209
 food, 9, 10, 15, 18, 28, 46, 53, 58, 75, 128, 186, 187,
 238, 266, 305, 306, 307, 424, 430
 food intake, 307

forebrain, 228, 251
 forests, xii, 99, 109, 111, 112
 formaldehyde, 246, 312, 336, 350, 359, 432
 formula, 147
 fragility, 137
 fragments, xii, 9, 59, 99, 100, 152, 153, 201, 228, 229, 234, 259, 261, 263, 281, 293, 312, 360, 376, 387, 392, 396, 397, 400, 403, 411, 414, 424
 France, 88, 218, 244, 357, 404
 freedom, 6, 7
 frequencies, 86, 400
 freshwater, ix, x, xiv, 36, 37, 38, 55, 173, 174, 185, 186, 190, 236
 fructose, xi, 45, 46, 47, 49, 52, 53
 fruits, 269, 366
 fulfillment, 350
 fungal infection, xv, 185, 190
 fungi, 189, 190, 203, 343
 fungus, xiv, xv, 165, 185, 187, 190, 198, 285, 343, 344
 furunculosis, 139
 fusion, 100, 243, 264, 320

G

gamete, 284
 gamma rays, 167
 ganglion, 265
 gases, 410
 gastric ulcer, 297
 gastrocnemius, 434
 gastrointestinal tract, xv, 75, 205, 207
 gastrula, 254, 277
 gastrulation, 228, 276
 gel, 59, 61, 197, 234, 289, 291, 293, 302, 321, 346, 375, 376
 gelatin zymography, 298, 385, 391
 gender, 269, 296, 429
 gene, xi, xiii, 57, 58, 59, 60, 61, 62, 63, 64, 66, 73, 75, 84, 117, 118, 120, 121, 122, 123, 124, 154, 158, 163, 208, 216, 219, 220, 225, 230, 234, 235, 238, 239, 240, 241, 242, 243, 245, 247, 249, 252, 256, 257, 258, 259, 260, 261, 266, 267, 268, 270, 284, 306, 314, 316, 317, 328, 334, 348, 370, 373, 374, 375, 376, 377, 378, 381, 384, 386, 390, 420
 gene amplification, 234, 270
 gene expression, 58, 63, 73, 75, 158, 208, 216, 219, 238, 245, 260, 266, 268, 314, 316, 317, 348, 373, 375, 377, 378, 386, 420
 gene promoter, 208
 gene silencing, 241
 generation, ix, x, xiv, 17, 28, 36, 38, 173, 175, 178, 179, 182, 228, 252, 282, 329, 358, 364

genes, ix, xi, xiii, 57, 58, 59, 60, 61, 62, 63, 65, 114, 117, 118, 120, 121, 124, 152, 153, 167, 208, 210, 226, 227, 229, 234, 235, 237, 238, 239, 241, 246, 247, 249, 250, 252, 256, 257, 259, 260, 261, 263, 266, 267, 268, 269, 270, 280, 281, 305, 306, 317, 348, 373, 377, 381, 390
 genetic alteration, 208, 308, 397
 genetic diversity, 118, 154
 genetics, 125, 154, 230, 249
 genital herpes, 168
 genital warts, 167, 168
 genome, xi, 57, 58, 60, 62, 63, 120, 167, 232, 233, 235, 238, 247, 250, 260, 261, 263
 genotype, 296
 genotypes, 152
 geography, 111, 115
 germ cells, 264
 gestation, 168, 351, 424, 426, 432, 433
 gingival, 78, 79, 301, 302
 gland, 25, 42, 158, 228, 230, 234, 240, 245, 249, 260, 293, 309, 326, 358, 377, 382, 388, 391, 396, 401, 413, 426
 glass, 128, 132, 158, 174, 175
 glia, 324, 362, 363, 364, 365, 368, 369
 glial cells, 318, 323, 325, 326, 363, 364, 370, 372
 glioblastoma, 371, 384, 394, 395, 396
 glioma, 273, 282, 384, 385, 390
 glucagon, 326
 glucose, xiv, xv, 157, 158, 159, 160, 162, 163, 175, 193, 197, 198, 199, 201, 356
 glucose metabolism, 163
 glucose tolerance, 158, 162
 glucose-induced insulin secretion, 162
 glutamate, 159, 287, 369
 glutathione, 428
 glycans, 282, 294
 glycerin, 412
 glycol, 269
 glycoproteins, 68, 74, 279, 282, 293, 320, 339, 340, 387, 415, 422
 glycosaminoglycans, 279, 289, 291, 292, 294, 299, 311, 348, 365, 374, 375, 376, 386
 glycoside, 73
 glycosylation, 58, 73, 75, 76, 380
 goblet cells, xi, xii, 67, 68, 71, 72, 74, 75
 gold, 296
 gonads, 101
 government, iv
 grades, 292
 gradient formation, 283
 granules, 68, 70, 224, 379
 granulomas, 285
 grass, 9, 13, 58

grasses, 2
 gray matter, 402
 grazing, 2, 13
 Great Lakes, 183
 Greece, 25, 218
 groundwater, 36, 55, 174
 groups, x, xiii, xiv, 27, 59, 78, 79, 90, 91, 95, 99,
 100, 111, 127, 129, 130, 131, 132, 133, 134, 135,
 136, 137, 154, 157, 158, 170, 207, 209, 211, 230,
 232, 234, 246, 257, 266, 278, 293, 298, 301, 302,
 303, 305, 306, 307, 308, 312, 315, 330, 336, 348,
 350, 353, 354, 356, 360, 371, 373, 379, 396, 398,
 400, 405, 406, 407, 409, 410, 412, 413, 416, 417,
 420, 421, 422, 424, 428, 429, 432, 433, 434
 growth factor, 241, 284, 301, 314, 319, 320, 323,
 326, 350, 353, 358, 363, 364, 368, 372, 376, 380,
 418
 growth rate, 4, 7, 10
 guidance, 265, 372
 guidelines, 175
 gut, 153, 206, 208
 gynecologists, 170

H

habitat, xi, 9, 10, 24, 28, 35, 38, 41, 45, 52, 53, 150,
 174, 186
 habitats, x, 2, 8, 13, 27, 29, 47, 173
 haematocrit, 133, 137, 139
 haemoglobin, 133, 136, 137, 139
 hair, xiv, 103, 105, 107, 151, 160, 162, 209, 306, 326
 hair follicle, 160, 162, 209, 326
 hairless, 106, 107
 handling, 137, 175
 haplotypes, 118
 harbors, 383
 hardness, 128
 healing, 55, 286, 297, 299, 306, 313, 319, 343, 356,
 362, 394, 405, 406, 413, 419, 425, 427, 428, 429,
 434
 health, x, 27, 128, 170, 182, 206, 291, 296, 300, 305,
 306, 331, 377, 387, 410, 416, 431
 health care, 377
 health services, 170
 health status, x, 27
 heart, xiv, 157, 158, 160, 161, 163, 175
 Heart, 158, 160, 163
 heart failure, 161, 163
 heart transplantation, 232, 334
 heart valves, 414, 415
 heat, 236, 249
 heat shock protein, 236
 heating, 196

heating rate, 196
 heavy metal, 183
 heavy metals, 36, 42, 54, 183
 height, 416
 hematologic, 168
 hematoma, 328
 hematopoietic stem cells, 400
 hematoxylin-eosin, 158, 169
 hemidesmosome, 312
 hemisphere, 9, 100
 hemorrhage, 352
 hemostasis, 282, 291, 347, 349
 hepatic failure, 400
 hepatic fibrosis, 296, 400
 hepatitis, 288, 400
 herbivorous, 153
 hernia, 432, 433
 herpes, 167, 168, 283
 herpes simplex, 283
 herpes virus, 167
 heterogeneity, 255, 379, 393
 high blood pressure, 410
 high-risk, 167
 hip, 401
 hip arthroplasty, 401
 histidine, 392
 histochemical, 169
 histochemistry, 74, 75, 293, 422, 424
 histological, 158, 161, 169
 histology, 160, 161, 358, 360, 423
 histone, 236, 246
 HIV, 168, 183, 288, 353
 HIV infection, 168
 HIV-1, 183, 288
 HIV-2, 288
 homeostasis, xiv, 52, 84, 157, 158, 162, 167, 227,
 278, 280, 282, 304, 329, 339, 403, 431
 homocysteine, 246, 318
 homogeneity, 90, 94, 313
 honey bees, 263
 hormone, xiv, 58, 62, 63, 64, 65, 66, 157, 158, 162,
 163, 229, 234, 249, 263, 279, 303
 hormones, xiv, 157, 158, 159, 161, 162, 163, 167
 hospital, 175
 host, 18, 73, 76, 206, 211, 214, 216, 232, 235, 280,
 282, 285, 341, 346
 HPV, 165, 167, 168
 HTLV, 353
 human, 165, 167, 174, 183
 human development, 323
 humans, 162, 163
 humidity, 238
 humoral immunity, 216

humus, xii, 99
 hyaline, 399
 hybrid, 68, 152, 245
 hybridization, 60, 124, 154, 237, 238, 243, 250, 253, 259, 268, 270, 283, 377
 hydro, 167
 hydrogen, 13, 410
 hydrolysis, 199, 200, 339
 hydroxide, 159
 hyperactivity, 409
 hyperglycemia, 430
 hyperplasia, 166, 301, 408, 429, 431
 hyperprolactinemia, 420
 hypersensitivity, 212
 hypertension, 409, 410, 414
 hypertensive, 161
 hyperthyroidism, 158, 161, 162, 163
 hypertrophy, xiv, 157, 158, 160, 161, 166, 284, 429
 hyperventilation, 410
 hypodermis, 293, 352
 hypoplasia, 166
 hypotension, 290
 hypothesis, 84, 231, 235, 240, 242, 250, 256, 261, 268, 287, 295, 308, 352, 392
 hypothyroidism, 158, 161, 163
 hypoxia, 227, 263, 402
 hypoxia-inducible factor, 227
 hysterectomy, 166

I

IBD, 294
 identification, 68, 100, 118, 124, 153, 154, 216, 227, 257, 260, 266, 270
 identity, 259, 268, 398
 IFN, 209, 211, 212, 213, 217, 218, 220, 285
 IL-13, 209
 IL-6, 213
 ileum, 68, 70, 71, 72
 illumination, 47
 image, 245, 342, 419, 427
 image analysis, 342
 images, 327, 346, 386
 immersion, 359
 immune function, 362
 immune response, x, xv, 27, 38, 183, 205, 206, 207, 209, 211, 212, 213, 214, 216, 219, 220, 221, 294, 330, 332, 334, 378
 immune system, 28, 36, 46, 128, 168, 288
 immunity, 54, 65, 138, 139, 165, 174, 212, 214, 216, 218, 285
 immunization, 300, 316, 331, 333, 336, 337
 immunocompromised, 344

immunoglobulins, 220
 immunohistochemistry, 75, 230, 241, 246, 307, 323, 329, 332, 340, 349, 351, 359, 364, 372, 378, 380, 387, 393, 405, 406, 407, 408, 416, 422, 426, 430
 immunological, 179, 182
 immunomarkers, 183
 immunomodulatory, 338, 402, 405
 immunoprecipitation, 344, 367
 immunoreactivity, 301, 303
 impacts, 283
 implementation, 169
 imprinting, 307
 in situ, 158, 165, 168, 182
 in situ hybridization, 237, 242, 250, 253, 268, 270
 in utero, 360, 422
 in vitro, x, xv, 37, 38, 39, 42, 46, 47, 50, 52, 53, 54, 55, 64, 65, 86, 174, 175, 205, 207, 212, 213, 214, 217, 218, 220, 221, 256, 265, 273, 277, 280, 290, 292, 295, 299, 311, 317, 319, 322, 324, 328, 332, 338, 342, 346, 347, 362, 363, 364, 365, 368, 375, 383, 391, 395, 399, 404, 416, 423
 in vivo, 40, 42, 49, 53, 58, 61, 163, 207, 212, 265, 281, 290, 295, 299, 317, 324, 347, 364, 367, 383, 387, 399, 432
 inactivation, 167
 incentive, 170
 incidence, 94, 165, 167, 168, 367, 374, 375, 385, 395, 412, 421
 incisor, xii, 77, 78, 79, 80, 81, 82, 83, 84, 85, 301, 302, 303
 inclusion, 233
 incubation, 175
 incubation period, 18, 22, 23, 24
 indentation, 103, 105, 107
 India, x, xi, xii, xiii, 12, 27, 28, 30, 35, 36, 37, 38, 42, 45, 46, 47, 54, 55, 99, 101, 105, 112, 113, 114, 115, 118, 124, 127, 128, 129, 138, 141, 142, 144, 148, 150, 154, 157, 158, 162, 173, 174, 182, 183, 186
 Indian, 152, 162, 174, 182
 indicators, 137, 285
 indices, 138, 242
 indirect effect, 366
 inducer, 378
 induction, xiv, 62, 157, 158, 161, 179, 213, 245, 249, 251, 299, 315, 320, 404, 418, 429, 435
 inefficiency, 280, 385
 infarction, 410
 infection, xv, 35, 128, 139, 140, 165, 167, 168, 183, 185, 187, 190, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 240, 274, 285, 288, 296, 329, 335, 342, 345, 353, 383

- infectious, 169
 infectious disease, 218
 inferences, 257
 inferior vena cava, 295
 infertility, 352, 420
 inflammation, xiv, 165, 166, 170, 209, 210, 273, 280, 282, 286, 287, 306, 328, 330, 331, 342, 348, 378, 379, 424, 426
 inflammatory bowel disease, 294
 inflammatory cells, 276, 285, 328, 333, 336, 350, 379, 398, 406, 412, 419, 424, 425, 427
 inflammatory disease, 330
 inflammatory mediators, 402
 inflammatory responses, 347
 ingestion, 246
 inhibition, 39, 47, 49, 52, 53, 54, 210, 219, 235, 242, 262, 270, 273, 282, 285, 287, 290, 318, 330, 339, 352, 368, 369, 375, 380, 385, 392, 394, 395, 396, 412, 414, 429
 inhibitor, 175, 267, 287, 290, 299, 313, 315, 339, 341, 344, 352, 353, 361, 364, 368, 369, 380, 384, 385, 394, 395, 396, 410, 412
 inhibitory, 153, 179
 inhibitory effect, 153, 179
 initiation, 168, 243, 261, 265, 395
 injection, 129, 160, 162
 injections, 299, 338, 360, 364, 372, 373
 injuries, 371
 injury, iv, 53, 275, 282, 286, 287, 288, 296, 297, 313, 319, 338, 349, 371, 398, 402, 406, 409, 429
 innate immunity, 138
 inoculation, 138, 213, 218, 220, 356
 inoculum, 194
 inositol, 287, 369
 insecticides, 150
 insects, 57, 58, 62, 66, 226, 240, 258
 insertion, 241, 249, 297
 insight, 36, 55, 315
 insulin, xiv, 157, 160, 162, 163, 229, 263, 301, 309, 326, 340
 integrin, 210, 215, 219, 276, 278, 281, 295, 311, 315, 320, 330, 344, 367, 383, 396, 402
 integrity, 276, 280, 312, 348, 384, 398, 415
 integument, 258, 293, 294, 312
 interaction, 55, 73, 89, 158, 163, 208, 233, 251, 257, 267, 284, 286, 287, 295, 298, 304, 311, 315, 322, 333, 335, 344, 345, 346, 362, 367, 369, 370, 380, 386, 397, 401
 interaction process, 322
 interactions, 10, 13, 73, 76, 211, 227, 228, 256, 273, 276, 279, 285, 304, 311, 312, 315, 320, 323, 330, 335, 339, 340, 363, 365, 373, 378, 379, 383, 387, 408, 432
 interdisciplinary, 169
 interface, 35, 211, 237, 271, 272, 279, 311, 322, 431
 interference, 46, 47, 49, 50, 51, 52, 53, 203, 227, 232, 235, 252, 300
 interneuron, 242
 interneurons, 242
 interphase, 167
 interrelationships, 115
 interstitial, 174, 182
 interval, 4, 39, 136, 137, 388
 intestinal tract, 242, 261
 intestine, xi, 67, 68, 71, 74, 75
 intima, 398
 intoxication, 400
 intramuscular, 129
 intramuscular injection, 79
 intramuscularly, xiii, 127, 129
 intravenously, 286, 290
 intron, 152
 invasive, 166
 invertebrates, 12, 14, 28, 38, 41, 42, 46, 52, 228, 237, 283, 294, 298
 investment, 191
 involution, 406
 iodine, 201
 ionizing radiation, 167
 ions, 53, 244, 410
 Iran, 186
 Iraq, 186
 irradiation, 305, 306, 416, 421, 434
 ischemia, 405, 428
 ischemic, 163
 Islam, 174, 183
 isolation, 196, 394
 Israel, 25
 issues, xiv, xvi, 151, 152, 170, 214, 228, 305, 306, 318, 328, 346, 374, 394, 403
 Italy, 219
 ITT, xiv, 157, 158, 160, 161

J

- Japan, 57, 59, 276
 jaw, 251
 jejunum, 68, 70, 71, 72, 73, 74
 joints, 103, 105, 107, 109, 111, 250, 356
 juveniles, 18, 23, 24, 92, 95, 264

K

- keratinocytes, 293, 312, 383
 keratoconjunctivitis, 207
 kidney, 86, 137, 138, 140, 207, 283, 337, 374, 375

kidneys, xv, 205, 207
 killing, 179, 182, 213, 285
 kinetic model, 239
 kinetics, 251, 404, 431
 King, 151
 knots, 88

L

labeling, 59, 231, 255, 292, 342, 363, 367, 373, 375, 382, 402
 labor, 429
 lactation, 307
 lactic acid, 142
 lakes, 128, 174
 lamella, 103, 109
 laminectomy, 371
 laminin-5, 278, 387, 388
 land, ix, 17, 25, 128
 landscape, 106
 Langerhans cells, 210, 219
 language, 11
 large intestine, xii, 67, 72
 larva, 248, 266, 267
 larvae, 128
 larynx, 378
 Latin America, 285
 laws, 84
 leaching, 174, 183
 leakage, 338
 learning, 248, 373
 left ventricular, 161
 leg, 142, 144
 leishmaniasis, xv, 205, 206, 207, 209, 210, 211, 212, 215, 216, 217, 218, 219, 220, 221
 lens, 262, 346
 Lepidoptera, 65
 leprosy, 274, 286, 410, 416
 lesions, 166, 167, 168, 169, 170, 208, 209, 211, 285, 292, 297, 299, 301, 313, 337, 338, 343, 349, 352, 355, 357, 361, 379, 380, 391, 398, 426
 leucine, 351, 427, 430
 leukemia, 217
 liberation, 376
 lice, 227
 life cycle, 8, 18, 23, 24, 25, 186
 life expectancy, 10, 11
 life span, 7, 18, 21, 23, 24, 284
 lifestyle, 410
 lifetime, 291
 ligament, xii, 77, 78, 79, 80, 81, 82, 83, 84, 85, 301, 403, 417
 ligand, 239, 287, 349

limitations, 169
 line, 6, 63, 93, 213, 219, 227, 241, 319, 370, 371, 374, 375, 376, 382, 383, 384, 385, 386, 388, 394, 396, 411
 linkage, 199, 200, 284
 links, 310, 312
 lipid metabolism, 241, 261
 lipid peroxidation, 405
 lipids, 58, 195, 198, 254, 259, 362
 liver, xv, 138, 139, 161, 163, 179, 205, 206, 207, 208, 210, 296, 400
 liver damage, 296
 liver enzymes, 139
 localization, 231, 241, 255, 256, 259, 261, 262, 278, 283, 303, 330, 342, 350, 351, 352, 376, 386
 locomotor, 288, 402
 locus, 153, 267
 longevity, 11, 238
 long-term memory, 287
 loss of appetite, 207
 Louisiana, 12
 low molecular weight heparins, 277
 low risk, 379
 LTD, 25
 luciferase, 227
 lumen, 69, 231, 292, 358, 398, 413, 426
 lung cancer, 378, 393
 lying, 21
 lymph, xv, 168, 205, 206, 207, 208, 210, 216, 217, 219, 221, 334, 379
 lymph node, xv, 168, 205, 206, 207, 208, 210, 216, 217, 219, 221, 334, 379
 lymphocyte, xiii, 127, 134, 137
 lymphocytes, 134, 137, 209, 211, 212, 213, 214, 328, 329, 332, 333, 374, 379
 lymphoid, 206, 211, 220, 221, 329, 332, 334
 lymphoid organs, 206, 329, 334
 lymphoid tissue, 211, 220, 221, 329
 lysine, 225
 lysis, 285

M

machinery, 227, 228, 233, 390
 macromolecules, 281, 375, 430, 433
 macrophage, 183
 macrophages, xv, 205, 206, 207, 208, 211, 212, 213, 214, 216, 218, 219, 220, 221, 285, 328, 333, 350, 353, 383, 398, 425
 magnesium, 55, 391
 magnetic resonance, 203
 magnetic resonance spectroscopy, 203

- maintenance, 31, 53, 235, 268, 294, 301, 304, 312, 327, 364, 397, 415
- major histocompatibility complex, 219, 221
- majority, 24, 62, 71, 88, 119, 199, 214, 288, 312, 360
- malaria, 341
- males, ix, 1, 4, 5, 64, 100, 101, 102, 111, 114, 152, 252, 293, 312, 335, 361, 422, 426, 429
- malignancy, 168, 169
- malignant, xiv, 165, 167, 170
- malignant melanoma, 280
- malignant tumors, 281, 306, 377
- malnutrition, 307
- maltose, 201
- mammal, 68, 73
- mammalian brain, 365
- management, 138, 150, 154
- mandible, 103, 104, 106, 108, 110
- manganese, 391
- mangroves, 95
- man-made, 128
- manners, 57
- mantle, 237
- manufacturer, 59
- Marfan syndrome, 276, 418
- marine mammals, 151
- marrow, xv, 205, 206, 207, 208, 210, 212, 329, 398, 400, 401, 403
- marsh, 2, 8, 12, 13, 14
- Maryland, 170
- mast cells, 328, 335, 379, 412, 425
- matrix metalloproteinase, 285, 299, 309, 347, 348, 358, 375, 377, 381, 382, 383, 394, 396, 397, 406, 407, 418
- maturation, 64, 65, 73, 75, 190, 229, 258, 360, 365, 367, 422, 431
- maxilla, 100
- MB, 154
- MCV, 131, 133, 136, 137
- measurement, xiii, 127, 139, 149, 254
- measures, 121, 137, 144, 207, 434
- meat, 194
- mechanical properties, 258, 284, 308, 313, 322, 327
- mechanical stress, 310, 414
- media, 194, 288, 318, 320, 348, 357, 372, 397, 398
- median, 29, 47, 207, 379
- medication, 410
- Mediterranean, 118, 124, 186, 206
- medulla, 339, 340
- meiosis, 114
- melanoma, 273, 280, 282, 375, 376, 381, 389, 391
- melting, 361
- membranes, 59, 75, 228, 246, 259, 261, 278, 320, 339, 377, 387, 388, 430
- memory, 248, 287, 370
- memory formation, 287, 370
- men, 301
- menstrual cycle, xiv, 165
- menstruation, 352, 355
- mesenchymal stem cells, 287, 399
- mesenchyme, 245, 246, 250, 359, 432
- mesentery, 330
- mesoderm, 233, 250, 263, 276
- mesothelial cells, 170
- messenger RNA, 63
- metabolic, 128, 162
- metabolic rate, 162
- metabolism, 163, 227, 229, 241, 256, 257, 261, 263, 308, 309, 354, 391
- metabolites, 432
- metalloproteinase, 241, 331, 377, 382, 390, 394, 424
- metals, 55, 183, 391
- metamorphosis, 249
- metastasis, 168, 273, 276, 281, 282, 306, 353, 358, 374, 375, 377, 380, 381, 382, 386, 388, 390, 391, 394, 423
- metastatic cancer, 380
- methanol, 30, 68, 195, 197
- methodology, 82, 202, 218, 267
- methylation, xv, 193, 200, 307, 395
- Mexico, 8
- MHC, 334
- mice, 211, 212, 221, 231, 237, 242, 274, 275, 279, 280, 284, 285, 286, 288, 291, 299, 308, 326, 328, 329, 330, 331, 332, 335, 339, 340, 341, 343, 345, 349, 358, 359, 361, 363, 364, 365, 366, 367, 370, 373, 389, 391, 398, 404, 417, 420, 425, 427, 429, 430
- microbial, 138, 179
- Microbial, 129, 138
- microenvironments, 326, 329
- microinjection, 228, 254, 435
- microorganism, xi, 41, 45, 68, 197
- microorganisms, 169, 170
- micro-organisms, 174
- microsatellites, 152, 153, 154
- microscope, 30, 47, 79, 101, 133, 159, 160, 168, 169, 175, 187, 269, 302, 327, 370, 372, 400, 415, 419, 427
- microscopy, 169, 228, 229, 231, 243, 244, 255, 257, 261, 264, 276, 277, 292, 295, 300, 310, 313, 317, 330, 333, 342, 345, 346, 356, 360, 367, 368, 376, 382, 385, 386, 388, 395, 410, 411, 414, 415, 418, 426, 430
- microtome, 269
- middle ear infection, 348

- migration, 84, 96, 210, 211, 214, 251, 271, 272, 273, 275, 278, 280, 281, 282, 288, 311, 312, 317, 318, 319, 320, 323, 324, 327, 328, 329, 330, 332, 334, 339, 340, 344, 345, 346, 348, 351, 365, 367, 368, 373, 375, 377, 382, 383, 384, 385, 387, 388, 389, 394, 395, 396, 398, 401, 404, 425, 432
 milligrams, 196, 197
 millipede, 100, 105, 111, 114, 115, 116
 mites, 141, 142, 150
 mitochondria, 228, 435
 mitochondrial, 153, 154
 mitochondrial DNA, xiii, 117, 118
 mitogen, 362, 363, 368
 mitosis, 167, 236, 385
 mitotic index, 236
 mitral valve, 415
 mixing, 88, 90
 MMA, 96, 312
 MMP, 241, 285, 297, 298, 299, 301, 302, 308, 309, 331, 348, 354, 358, 360, 361, 362, 375, 381, 382, 383, 384, 387, 388, 390, 391, 396, 406, 418, 422, 423, 424
 MMP-2, 241, 297, 298, 299, 301, 302, 308, 309, 331, 348, 354, 358, 360, 361, 375, 381, 383, 384, 390, 391, 396, 418, 422, 423, 424
 MMP-3, 348
 MMP-9, 285, 298, 301, 308, 309, 331, 348, 354, 360, 381, 383, 384, 390, 391, 418, 423, 424
 MMPs, 298, 299, 301, 302, 303, 309, 348, 355, 357, 360, 361, 375, 381, 383, 384, 390, 391, 394, 395, 396, 406, 418, 422, 423
 mobility, 182, 229, 356, 368
 model, xiii, xvi, 3, 15, 58, 78, 82, 117, 182, 205, 206, 207, 210, 213, 216, 218, 219, 226, 232, 239, 251, 266, 269, 271, 275, 284, 289, 290, 291, 292, 294, 295, 299, 311, 316, 317, 328, 331, 333, 334, 335, 336, 337, 340, 341, 347, 349, 352, 353, 362, 367, 371, 379, 385, 391, 404, 406, 418, 423
 model system, 226, 266, 284
 modeling, 86
 modelling, 216
 models, 207, 210, 212, 213, 236, 266, 291, 331, 336, 347, 352, 390, 402
 modulations, 235
 modules, 310
 modulus, 284, 308, 310
 moisture, xii, 18, 99
 mole, 351
 molecular dynamics, 311, 392
 molecular markers, xiv, 151, 152
 molecular mass, 257, 390
 molecular weight, 252, 277, 289, 290, 300, 315, 347, 357, 397
 molecules, xi, 28, 54, 57, 58, 68, 179, 208, 210, 216, 233, 250, 254, 281, 282, 284, 287, 289, 291, 303, 304, 309, 310, 311, 315, 320, 323, 324, 330, 332, 335, 340, 346, 353, 357, 358, 367, 369, 370, 371, 375, 376, 377, 380, 396, 397, 402, 417, 418, 427, 431, 432
 mollusks, 14
 monoclonal antibody, 211, 277, 367, 374
 monocytes, 134
 monolayer, 168, 175, 325, 383, 395
 monosaccharide, xv, 193, 198, 199
 morbidity, 38, 333, 403, 421
 morphogenesis, 225, 233, 236, 246, 250, 264, 272, 274, 275, 278, 283, 358, 359, 371, 426
 morphological, 169, 170
 morphology, ix, xiv, 12, 42, 85, 100, 157, 158, 159, 161, 162, 163, 166, 183, 194, 230, 269, 281, 292, 322, 326, 332, 345, 359, 364, 366, 399, 413, 415, 432
 morphometric, 75, 316, 331, 354, 356, 416
 mortality, xv, 2, 4, 8, 10, 35, 38, 47, 131, 169, 185, 190, 242, 333, 383, 385, 389, 398, 410
 mortality rate, 4, 8, 11, 190, 383, 389
 mosaic, 231, 280
 mothers, 307
 motif, 230, 261, 310, 385
 motives, 257
 motor behavior, 372
 mouse, 163
 movement, 78, 83, 269, 278, 291, 293, 301
 mRNA, 60, 62, 63, 64, 209, 211, 212, 213, 214, 233, 241, 252, 253, 256, 261, 270, 279, 282, 307, 314, 316, 326, 333, 336, 357, 358, 381, 385, 386, 411, 416
 MS, 154
 mtDNA, 118, 120, 124, 153
 mucin, 71, 72, 73, 75
 mucoid, 357
 mucosa, 75, 76, 306, 315, 387
 mucus, 68, 71, 72, 73, 75, 153, 168
 multicellular organisms, 232
 multidimensional, x, 27
 multiplication, 167, 214
 multipotent, 323, 325, 326
 muscle, xiv, 157, 158, 160, 161
 muscle cells, 161
 muscles, 161, 291, 298, 320, 321, 328, 404
 muscular dystrophy, 328
 mushrooms, 150
 mutagenesis, 242
 mutant, 231, 239, 241, 249, 251, 252, 267, 311, 328, 370
 mutant proteins, 370

mutation, 118, 167, 168, 230, 241, 249, 252, 283, 370, 392, 397
 mutations, 167
 myelin, 324, 325
 myeloid cells, 211
 myoblasts, 320, 404
 myocardial infarction, 410
 myocyte, 161, 163
 myofibroblasts, 326, 405, 412, 416, 425
 myogenesis, 320, 321
 myopathy, 328, 355
 myosin, 158, 262, 384

N

NaCl, 129, 175, 195, 336, 353, 361, 411, 420
 naming, 144
 nanoparticles, 294
 nanotube, 322
 National Research Council, 215
 natural, xiv, 158, 173, 179
 natural habitats, 173
 necrosis, 129, 217, 332, 402
 needles, 168
 neoplasia, 166, 168
 neoplasm, 382, 387, 388, 418
 neoplastic, 169, 170
 neovascularization, 232, 347, 348
 nephron, 85
 nerve, 85, 410, 416
 nervous system, 236, 237, 265, 266, 287, 317, 318, 324, 325, 326, 363, 367, 369
 network, 224, 225, 236, 256, 281, 284, 314, 327, 340, 342, 367, 419
 neural development, 265, 287
 neuralgia, 207
 neuroblasts, 248, 266
 neurodegenerative disorders, 370
 neurogenesis, 237, 265, 266, 372
 neuronal apoptosis, 338
 neuronal cells, 265
 neurons, 237, 265, 287, 317, 318, 323, 324, 325, 326, 362, 363, 364, 365, 366, 368, 369, 370, 371
 neuropeptides, 58
 neutral lipids, 254
 neutrophil, 132, 133
 neutrophilia, 137
 neutrophils, 137, 330, 338, 379, 405
 New England, 13, 14
 new media, 282
 Nigeria, 11
 nitrate, 183

nitric oxide, xiv, 173, 174, 183, 208, 218, 219, 277, 313, 315, 385, 410, 414
 nitric oxide (NO), xiv, 173
 nitric oxide synthase, 174, 208, 385, 410
 Nitrite, 182
 nitrogen, xiv, 2, 13, 59, 163, 173, 174, 195, 213, 348, 408, 420, 423
 NMR, xv, 193, 197, 198, 199, 200, 201, 203
 NO, xiv, 173, 174, 175, 178, 179, 181, 182
 nodes, 206, 207, 210, 221, 334
 nodules, 107
 non-invasive, 154
 normal, xiv, 157, 158, 160, 166, 167, 179
 normal development, 265, 426
 norms, 175
 North Africa, 206
 North America, 150, 191
 notochord, 276
 nuclear, 153
 nuclear genome, 120
 nuclear magnetic resonance, 203
 nuclei, 170, 248, 261, 304, 318, 320, 367, 430
 nucleic acid, 59, 195, 198, 270
 nucleotides, 120, 121
 nucleus, 166, 231, 410
 nursing, 266
 nutrient, 129, 130
 nutrients, 28, 46, 58, 88, 94, 169, 432
 nutrition, 75, 183, 248, 266

O

obesity, 412
 observations, 5, 8, 13, 19, 22, 23, 30, 97, 101, 281, 298, 357
 obstruction, 295, 355
 occlusion, 349
 oil, 303, 423, 424
 oligosaccharide, 68
 oncogene, 167
 oncogenes, 167, 383
 oocyte, 62, 64, 65, 190, 257, 261
 oogenesis, 59, 61, 256
 opportunities, 191
 optical, 169
 optical microscopy, 243, 244, 410
 oral cavity, 228
 order, xii, 11, 28, 62, 90, 99, 100, 101, 111, 190, 194, 195, 196, 198, 199, 201, 202, 212, 228, 231, 232, 233, 236, 245, 249, 254, 260, 263, 267, 284, 292, 312, 317, 322, 326, 334, 338, 339, 340, 345, 346, 347, 352, 367, 370, 372, 373, 378, 381, 383, 385, 399, 403, 416, 420

organ, xiii, 69, 99, 114, 115, 158, 163, 241, 245, 268, 280, 288, 309, 332, 340, 358, 391, 400, 417, 421
 organelles, 85, 166, 170, 228
 organic, 139
 organic matter, xii, 99
 organism, 169, 407
 orientation, 263, 276, 285
 oryx, 121, 124, 154
 oscillation, 3, 7
 oscillations, 406
 osmium, 79
 osmotic pressure, 186
 ossification, 361
 otitis media, 348
 ovariectomy, 279, 430
 ovaries, 58, 257, 261
 overproduction, 281
 overtime, 372
 oviduct, 83, 84, 85
 oxidation, 327, 415
 oxidative damage, 428
 oxidative stress, 330, 331, 428
 oxide, 183
 oxygen, 13, 29, 47, 90, 91, 94, 128, 182, 187, 191, 194, 213, 227, 402
 oxygen consumption, 191

P

Pacific, 13
 pain, 291, 352, 355
 Pakistan, 35, 54, 186, 190
 pancreas, 162
 pancreatic, 162, 163
 pannus formation, 336
 Pap smear, 168, 169
 Papillomavirus, 165
 paradigm, 277
 parallel, 147, 232, 241, 279, 288, 299, 307, 314, 399, 405, 415
 paralysis, 372
 parameter, 3, 7, 11, 133, 179
 parameters, xiii, xiv, 2, 3, 4, 7, 12, 60, 81, 83, 101, 127, 128, 131, 133, 135, 136, 137, 139, 173, 174, 179, 247, 284, 285, 294, 360, 400, 407, 419
 parasite, xv, 73, 76, 205, 207, 209, 210, 211, 213, 214, 216, 217, 220, 221, 341, 342, 345
 parasites, xiv, 165, 170
 parenchyma, 338, 400, 402, 422
 parenchymal cell, 410
 parents, xv, 185, 189
 partial thromboplastin time, 349
 particles, x, 37, 42, 175, 182

pathogenesis, 139, 206, 287, 314, 336, 340, 341, 342, 345, 370
 pathogenic, 128
 pathogenic agents, 128
 pathogens, 28, 130, 137, 218, 240
 pathologist, 169
 pathology, 169, 271, 274, 275, 277, 291, 309, 337, 339, 366, 429
 pathophysiology, 287, 314, 330
 pathways, 218, 227, 235, 258, 259, 272, 277, 282, 304, 317, 324, 328, 344, 362, 363, 367, 368, 369, 378, 391, 396
 patients, 162
 PBMC, 212, 213, 214, 220
 pelvis, 421
 penis, 421, 432
 peptides, 240, 241, 273, 281, 284, 304, 368, 377, 382, 383, 388, 390, 392, 396
 PER, 130
 performance, 3, 97, 186, 187, 197
 perfusion, 79
 pericardium, 328
 periodontal, xii, 77, 78, 79, 80, 81, 82, 83, 84, 85, 301
 peripheral blood, 208, 212, 216, 220, 329, 400
 peripheral blood mononuclear cell, 220
 peripheral nervous system, 265, 318, 324, 325, 326, 363
 peritoneal cavity, 335
 peritoneum, 335, 355
 peritonitis, 330
 permeation, 197
 permission, iv, 114
 peroxidation, 405
 peroxynitrite, 174, 179
 personal computers, 124
 pest management, 150
 pesticide, xiii, 36, 39, 127, 128, 129, 130, 131, 132, 134, 135, 136, 137
 pesticides, xiii, 127, 128, 139
 pests, 17, 18, 25
 phagocyte, 138, 210, 211, 214, 216
 phagocytic, xiv, 173, 174, 175, 176, 179, 180, 183
 phagocytosis, x, xi, xiv, 37, 38, 39, 42, 43, 45, 173, 174, 175, 182, 183, 208, 216
 pharmaceuticals, 128
 phenocopy, 265
 phenothiazines, 235
 phenotype, 167, 215, 228, 230, 235, 249, 257, 277, 280, 281, 292, 325, 364, 371, 386, 397, 399, 401, 426, 429
 Philadelphia, 139, 140
 phorbol, 182

- phosphatidylserine, 435
phospholipids, 254, 391
phosphorylation, 58, 344, 363
phthalates, 424
phylogenetic tree, 120, 124
phylum, 95
physical activity, 373, 409, 410
physical environment, 14
physical properties, 399
physicochemical properties, 289
physiological, 128, 129, 137, 138, 166
physiological correlates, 138
physiological factors, 166
physiology, 2, 58, 241, 275, 282, 339, 358, 391
physiopathology, 354
phytoplankton, 94
phytotherapy, 434
pigmentation, 194, 258
pigs, xi, 67, 68, 71, 72, 74, 75
pilot study, 154
pituitary, 139
PKD, 138
placenta, 272, 278, 280, 351, 432, 433
placental barrier, 433
planning, 317
plants, xi, 18, 45, 186, 232, 235, 240, 268, 371, 395
plaque, 286, 292
plasma, 55, 209, 259, 286, 290, 292, 310, 320, 329, 330, 341, 342, 349, 374, 423, 434
plasma cells, 209, 329
plasma membrane, 259, 320, 330
plasminogen, 357
plasticity, 287, 327, 370, 372
plasticizer, 424
plastics, 424
platelet aggregation, 282
platelet count, 296
platelets, 276, 282, 349
play, 162
Pleurotus ostreatus, xiii, 141, 148
plexus, 209
point mutation, 283
poison, 174
poisoning, 182
polarity, 83, 230, 245, 276, 312
polarization, 262, 276, 327
polarized light, 169
polarized light microscopy, 310, 313
pollen, 229, 238
pollination, 269
pollinators, 269
pollutants, 139
pollution, 36, 46, 53, 55, 128, 179
polyacrylamide, 291
polycyclic aromatic hydrocarbon, 167
polymer, 199, 287, 289, 311
polymerase, 216, 218, 219, 234, 248, 378, 420
polymerase chain reaction, 216, 218, 219, 248, 378, 420
polymerization, 231, 262, 287, 325, 345
polymers, 287, 290, 317, 338, 367, 431
polymorphism, 118
polymorphisms, 208, 268
polyploidy, 65
polypropylene, 158
pools, 186
poor, 153, 287, 342, 395
population, ix, x, xi, xiii, 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 25, 28, 30, 32, 37, 38, 39, 42, 46, 85, 118, 127, 131, 132, 133, 134, 136, 137, 138, 151, 154, 169, 182, 186, 206, 207, 211, 298, 323, 324, 325, 326, 328, 334, 350, 355, 364, 365, 366, 368, 392, 400, 403, 428
population density, 5, 9
population size, 154
Porifera, 298
Portugal, 10, 14, 432, 433
positive correlation, 211, 213, 296, 381, 390, 418
positive macrophages, 425
post-transcriptional regulation, 307
potassium, 201
poultry, 173
power, 4
precipitation, xv, 2, 9, 193, 195, 199, 348
predation, xi, xv, 45, 182, 185, 191
prediction, 15
pregnancy, 278, 360, 417, 427, 429, 430, 432, 433
prejudice, 170
pressure, 10, 186, 247, 256, 284, 315, 409, 410, 414, 415
preterm delivery, 430
prevention, 162, 166, 169, 170, 294
preventive, 165, 166, 170
PRI, 332
primary brain tumor, 395
primary function, 389
primary tumor, 280, 385, 386
priming, 212, 218, 221
probability, 6, 7
probe, 59, 85, 234, 241, 287, 295, 369, 370, 397, 398
production, ix, xiv, xv, 1, 2, 3, 4, 8, 9, 11, 12, 13, 14, 15, 23, 25, 49, 64, 65, 83, 88, 139, 150, 162, 179, 185, 190, 193, 194, 197, 209, 212, 213, 214, 216, 217, 219, 240, 251, 265, 270, 276, 285, 294, 298, 315, 319, 331, 332, 335, 336, 357, 383, 392, 402, 407, 410

productivity, 2, 11, 190
 progesterone, 406, 420, 426, 430, 431
 prognosis, 349, 378, 381, 390, 393, 394, 395
 program, 4, 8, 60, 90, 119, 124, 404, 435
 programming, 360, 422
 pro-inflammatory, 294, 330
 project, 103, 105, 109, 268, 270, 321, 341, 428
 prolactin, 420
 prolapse, 417
 proliferation, xv, 35, 166, 167, 168, 205, 217, 227,
 246, 255, 264, 266, 275, 278, 283, 288, 304, 311,
 312, 314, 317, 318, 320, 321, 325, 327, 329, 332,
 339, 340, 351, 356, 358, 366, 367, 371, 373, 375,
 378, 380, 385, 388, 389, 394, 396, 398, 399, 400,
 401, 404, 425, 426, 430
 promoter, 167, 168, 208, 239, 240, 245, 252, 260,
 267, 306
 properties, 84, 86, 128, 235, 258, 274, 278, 283, 284,
 287, 289, 293, 308, 310, 311, 312, 313, 321, 322,
 326, 327, 349, 353, 369, 399, 403, 431
 prophylactic, 137
 prophylaxis, xiv, 165, 170
 prostate, 241, 301, 303, 306, 307, 309, 357, 358,
 359, 360, 380, 385, 391, 394, 406, 407, 408, 413,
 416, 422, 423, 424
 prostate cancer, 301, 380, 385, 394, 414, 423
 prostate gland, 309, 391
 proteases, 289, 290, 322, 339, 345, 357, 389
 protective role, 214
 protein, 139, 167, 168
 protein kinase C, 368
 protein sequence, 233, 268
 protein synthesis, 58, 255, 364
 proteinase, 339
 proteins, 63, 65, 68, 78, 79, 139, 159, 167, 169, 195,
 198, 227, 228, 230, 233, 235, 245, 249, 250, 255,
 256, 259, 261, 270, 271, 272, 273, 277, 287, 288,
 291, 293, 296, 299, 304, 309, 310, 319, 320, 324,
 330, 334, 339, 346, 348, 362, 363, 368, 370, 379,
 384, 388, 404, 411, 416, 417
 proteoglycans, 279, 281, 285, 299, 332, 351, 357,
 372, 373, 374, 376, 386, 403, 410, 413, 414, 427,
 430, 431
 proteolysis, 281, 357, 394
 proteolytic enzyme, 383, 396
 proteome, 238
 prothrombin, 349
 protocol, xiii, 117, 119, 310, 326, 327, 373, 392, 432
 protocols, 219, 316, 399, 403
 proto-oncogene, 167
 PSD, 363
 psychological stress, 425
 psychosis, 235

public, 170
 public health, 35, 170, 206, 291, 296
 pulp, 78, 83, 85, 139, 153, 211, 323, 329
 pulp mill, 139
 pulse, 249, 284
 purification, 198
 pyruvic, 163

Q

qualitative differences, 343
 quality of life, 354

R

radiation, 124, 167, 236, 305, 306, 394, 421
 Radiation, 421
 radiation therapy, 394
 radicals, 179, 182
 radiography, 327
 radiotherapy, 166, 305, 306, 416, 421
 radula, 12
 rainfall, 109
 range, 2, 4, 6, 7, 9, 24, 28, 46, 88, 102, 129, 182,
 185, 191, 257, 294, 365, 372, 401
 RANTES, 211
 rat, xiv, 157, 158, 159, 160, 161, 162, 163
 rats, 158, 159, 160, 161, 162, 163
 reactions, 153, 169, 197, 219, 236, 257, 317, 355,
 407, 413, 431
 reactive nitrogen, xiv, 173, 174
 reactive oxygen, 213
 reactivity, xi, 67, 71, 72, 73, 75, 350
 reading, 233, 260
 reagent, 175
 reagents, 321, 329
 real time, xi, 57, 58, 60, 61, 62, 251, 308, 317, 372,
 373, 378, 381, 390
 reality, 154
 reason, 252, 414
 Receiver Operating Characteristic (ROC), 353
 receptors, 49, 65, 78, 208, 215, 228, 229, 235, 278,
 282, 283, 303, 311, 312, 332, 340, 341, 343, 362,
 363, 369, 376, 383, 396, 416
 recognition, x, 28, 37, 38, 39, 42, 46, 179, 274, 283,
 320, 346, 379
 recommendations, iv
 reconstruction, 300, 378, 406
 recovery, xiv, 30, 32, 35, 136, 173, 174, 179, 288,
 338, 398, 421
 rectosigmoid, 355
 rectum, xii, 67, 68, 70, 72, 74
 recurrence, 281, 382, 388, 418

- recycling, 2
 red blood cell, 158
 red blood cells, 158
 refraction index, 197
 regenerate, 313
 regeneration, 232, 287, 308, 321, 327, 328, 338, 350, 399, 402, 404, 410, 416
 region, xi, xii, 15, 35, 46, 54, 87, 88, 89, 90, 94, 95, 105, 106, 112, 120, 124, 147, 152, 186, 200, 206, 208, 229, 232, 233, 236, 242, 247, 250, 251, 252, 255, 256, 260, 262, 263, 264, 265, 267, 297, 300, 302, 306, 318, 327, 361, 370, 379, 413, 430
 regression, 4, 5, 8, 332, 348, 352
 regression equation, 5, 8
 regulation, 9, 25, 58, 64, 66, 161, 224, 225, 229, 236, 239, 249, 257, 258, 265, 282, 307, 309, 328, 335, 377, 378, 396
 regulators, 225, 227, 262, 264, 268, 282, 330, 339, 383
 rejection, 273, 281, 334, 402
 relationship, xii, 4, 6, 9, 77, 83, 85, 118, 210, 256, 274, 284, 295, 301, 334, 356, 398
 relaxation, 315, 408, 417
 relevance, 209, 256, 260, 262, 280, 292, 388
 reliability, xiv, 154, 173
 remediation, 179
 remodeling, 161
 remodelling, 227, 332, 346, 374, 376
 renal failure, 338, 410
 repair, 297, 299, 300, 313, 319, 398, 403, 405, 413, 419
 reparation, 194, 336
 replacement, 163, 279, 303, 309, 403
 replication, 167, 208, 246
 repressor, 240, 242
 reproduction, 2, 9, 10, 14, 25, 64, 190, 191, 238, 418
 reproductive organs, 12
 resection, 393
 reserves, 28, 46, 64
 reservoir, 183
 residues, 38, 49, 52, 68, 71, 341, 392
 resistance, 35, 140, 166, 211, 216, 218, 235, 301, 322, 435
 resolution, 248, 264
 resources, 154
 respect, 49, 53, 149, 211, 228, 257, 260
 respiratory, 263, 410, 435
 respiratory rate, 410
 responsiveness, xi, 57, 139, 182, 326, 342
 restenosis, 286, 398
 restriction enzyme, 307
 retardation, 412
 retention, 293
 reticulum, 64, 287, 369
 retina, 252, 255, 256, 367
 retinal detachment, 367
 retirement, 291
 retroviruses, 354, 383
 RFLP, 154
 rheumatoid arthritis, 394
 ribosomal, 153
 ribosomal RNA, 124
 rights, iv
 rings, 100, 315
 risk, xi, 45, 167, 168, 169, 170, 174, 208, 217, 219, 294, 379, 383, 409, 410
 risk factors, 168, 170, 217
 RNA, 59, 124, 211, 225, 232, 234, 235, 238, 252, 255, 256, 270, 279, 296, 348, 377, 390, 403, 420
 RNA processing, 255
 RNAi, 227, 238, 249
 robustness, 253
 rodent, 158
 room temperature, 59, 68, 79, 175, 195
 Royal Society, 12, 216, 217, 218, 221
 rubber, 18
 runoff, 38
 rural, 173
 rural areas, 219, 285

S

- safety, 35
 saline, 129, 175
 salinity, xii, 4, 6, 9, 87, 90, 91, 94, 191
 saliva, 240
 salivary glands, 234, 240, 245, 249, 260, 283
 salmon, 138, 139, 140, 194
 salt, 2, 8, 10, 12, 13, 14, 191, 410
 sample, 131, 133, 151, 153, 159, 169, 170
 sampling, ix, xiv, 4, 5, 6, 7, 8, 89, 90, 91, 94, 133, 137, 151, 152, 155, 188, 216
 SAP, 35, 42, 54, 182
 saponin, 217
 satellite, 79, 83
 saturation, 187
 Saudi Arabia, 117, 151, 185, 186, 190, 191
 scanning electron microscopy, 114, 270, 322, 345
 scholarship, 347
 scleroderma, 316, 332, 333, 337
 sclerosis, 300, 313, 314, 316, 331, 333, 336, 337
 screening, 53, 169, 182, 366, 376
 search, 233, 251, 253, 294
 searches, 169, 263
 searching, 296
 seasonality, 3

- secrete, 374
secretion, 58, 162, 163, 281, 282, 292, 314, 320, 322, 330, 360, 368, 371, 383, 388, 396, 411, 416, 423, 426
sediment, 2, 3, 9, 14, 35, 36, 55, 174, 182, 259
sediments, 10, 36, 182
seed, 203, 383, 399
seeding, 431
segregation, 325
self-assembly, 430
self-fertilization, 18
self-regulation, 239
seminal vesicle, 78, 426
seminiferous tubules, 264
senescence, 167, 238, 301, 303, 355
sensitivity, 72, 163, 182, 235, 317
sensitization, 182
sensors, 84
separation, 206
sequencing, 124, 152, 234, 241, 257
series, 129, 168
serine, 289, 344, 357, 363, 411
serology, 218, 220, 221
Sertoli cells, 264
serum, xiv, 68, 139, 157, 158, 159, 161, 163, 208, 212, 218, 296, 322, 325, 326, 335, 338, 353, 360, 365, 368, 374, 394, 395, 397, 401, 405, 407, 420, 424, 431, 434
serum albumin, 68, 335
services, 170
seta, 142, 147
severity, 207, 209, 354, 355
sewage, 138
sex, ix, 1, 4, 57, 152, 153, 154, 252, 280, 339, 429, 430
sex hormones, 280, 430
sex ratio, 4
sex steroid, 429
sexual activity, 168
sexually transmitted disease, 168
sexually transmitted diseases, 168
SGOT, xiv, 157, 158, 160, 161
SGP, 436
shape, xii, 14, 22, 68, 99, 107, 114, 124, 161, 163, 170, 194, 292, 382, 419, 427
shares, 111, 237, 249
sharing, 231
shear, 86
sheep, 152, 153
shelter, 10
shock, 236, 249
shoot, 268
shortage, 400
short-term, 129
shrimp, 42, 43, 88, 347
SI, 154
side effects, 294, 305, 306, 310, 341, 407
signal transduction, 246, 317, 350, 377
signaling pathway, 78, 254, 259, 277, 295, 344, 357, 362, 363, 367, 369, 391, 396
signalling, 227, 368, 376, 418
signals, xiv, 84, 165, 197, 198, 199, 203, 224, 228, 253, 282
significance level, 90
signs, 11, 161, 206, 207, 209, 330, 355, 430
silica, 197, 348
silk, 399
silver, 347
simulation, 311
sinus, 263
sinusitis, 335
siRNA, 241, 358, 383, 388, 396
sites, 159
skeletal muscle, 163, 320, 321
skeleton, 84, 318
skin, xiv, xv, 86, 138, 157, 158, 159, 161, 168, 205, 206, 207, 208, 209, 210, 211, 214, 216, 217, 219, 221, 297, 299, 300, 313, 316, 327, 331, 333, 336, 352, 405, 412, 419, 425, 427, 428, 429
sleep apnea, 355
small intestine, xii, 67, 72, 73, 74, 75
smoking, 167, 183
smooth muscle, 85, 86, 231, 284, 286, 292, 315, 318, 323, 324, 325, 357, 358, 359, 360, 398, 403, 405, 408, 413, 414, 416, 421, 425, 426, 427, 429, 435
smooth muscle cells, 85, 284, 292, 318, 323, 324, 325, 359, 398, 408, 426, 427, 429, 435
snake venom, 352, 394, 434
snakes, 247, 256
social group, 170
sodium, x, xi, xiv, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 132, 159, 173, 174, 175, 176, 177, 178, 179, 195, 203, 292, 353, 423, 431
sodium dodecyl sulfate (SDS), 431
sodium hydroxide, 159
software, xiii, 60, 117, 119, 257, 303, 306, 311, 333, 346, 356, 374, 377, 400, 407, 421, 432
soil, xii, 18, 22, 24, 99, 100, 115, 271, 272
soil particles, 100
soils, 183
solid tumors, 375
solubility, 197
Somalia, 186
somatic cell, 264
South Africa, 14, 112

- Southern blot, xi, 57, 59, 60, 234, 251
space, 53, 112, 229, 264, 422, 424
spatial location, 6
specialists, 355
speciation, 284
species, ix, x, xii, xiii, 2, 3, 8, 9, 10, 11, 12, 15, 17, 18, 19, 21, 22, 23, 24, 35, 37, 41, 52, 53, 57, 58, 64, 67, 69, 70, 71, 72, 73, 87, 90, 91, 93, 94, 95, 100, 101, 106, 111, 112, 114, 115, 116, 117, 118, 120, 121, 124, 141, 142, 144, 148, 149, 150, 151, 152, 154, 173, 183, 186, 190, 194, 198, 206, 232, 243, 247, 256, 260, 269, 270, 274, 283, 288, 298, 309, 338, 340, 432, 433
spectroscopy, 203
spectrum, 9, 10, 198, 199, 200, 201, 210, 216
speed, 78, 79, 88, 191, 265, 302, 388
sperm, 100, 243, 283
spermatogenesis, 265
spinal cord, 237, 242, 246, 275, 288, 338, 372, 402
spinal cord injury, 275, 338, 402
spine, 103, 105, 107, 111, 112
spleen, xv, 84, 205, 206, 207, 208, 210, 211, 218, 221, 329
sponge, 298
Spring, 65, 78, 85
squamous cell, xiv, 165, 281, 383, 386, 387
squamous cell carcinoma, 281, 383, 386, 387
Sri Lanka, xii, 99
stability, 167, 183, 253
stages, xiv, 165, 166, 167, 168
standard deviation, 5, 6, 31, 32, 39, 92
standard error, 133, 176, 425
standardization, 101
standards, 201
statin, 405
statistics, 8, 14
stem cell lines, 288
stem cells, 68, 237, 275, 287, 323, 326, 394, 399, 400, 402
stent, 286
sterile, 30, 38, 47, 130, 175, 238, 252, 319, 330
sternalis, 149
steroids, 279, 310, 407, 429
stigma, 225
stimulant, 309
stimulus, 235, 269, 274, 277, 284, 298, 314, 342, 416
stock, 13, 175, 317
stomach, 379
storage, 238, 259
strain, xiii, 127, 129, 328, 339, 342, 345, 391
strains, xiii, 127, 129, 138
strategies, 191, 287, 330, 379, 403, 404
strategy, 35, 179, 267, 399
stratification, 6, 94, 174
strength, 84, 153, 283, 301, 308, 389
stress, x, xi, xii, xiv, 27, 34, 36, 37, 52, 53, 99, 127, 128, 137, 140, 151, 163, 173, 182, 224, 227, 235, 257, 308, 310, 330, 331, 378, 384, 407, 414, 425, 428, 431
stress granules, 224
stressors, 138, 139, 236
stretching, 24
stroke, 410
stroma, 278, 279, 301, 303, 307, 315, 355, 357, 358, 359, 360, 380, 387, 407, 408, 413, 414, 418, 426, 427, 430
stromal cells, 314, 315, 360, 400, 403, 413, 418
structural changes, 220, 284, 432
structuring, 12
subcutaneous injection, 330, 335
subgroups, 420, 430
substances, 128
substitution, 120, 125, 207, 336, 356, 400
substrates, 159, 278, 317, 361
sucrose, 194, 238, 254
Sudan, 186
sugar, 49, 52, 195, 298
sulfuric acid, 196, 197
summer, xii, 8, 9, 35, 99, 174
Sun, 65
superiority, xiii, 117
superoxide, 174, 179
supply, 29, 47, 187, 352, 402
suppression, 41, 162, 176, 179, 206, 216
suppressor, 167
surface layer, 168
surface water, 128
surgical resection, 393
survey, 91
survival, x, xi, 27, 45, 54, 216, 220, 245, 276, 312, 317, 319, 325, 332, 341, 352, 363, 366, 369, 371, 379, 389, 435
survival rate, 389
susceptibility, 128, 138, 182, 208, 211, 220, 339
suture, 109, 297
swamps, 128
Switzerland, 276
symbols, 30
symptom, 214, 352
symptoms, 206, 207, 212, 338, 355, 408
synapse, 363, 372
synchronization, 304
syndrome, 276, 355, 418
synovial membrane, 336
synovial tissue, 335, 336, 356

synthesis, xi, 25, 57, 58, 62, 64, 167, 236, 242, 255, 263, 270, 274, 277, 279, 284, 291, 295, 297, 298, 299, 314, 327, 333, 335, 357, 364, 368, 376, 377, 379, 380, 391, 414, 417, 429, 431
systemic sclerosis, 300, 314

T

T cell, 212, 213, 218, 219, 221, 328, 332, 334, 340
tachycardia, 161
tags, 268
tamoxifen, 413
tanks, xiv, 174, 185, 187, 189, 190
target organs, 209
targets, 218
taxonomy, 111, 115, 194
technical assistance, 73, 84, 114
teeth, xii, 77, 78, 79, 82, 83, 103, 105, 107, 109, 111, 113, 147, 149, 301, 302
telomeres, 167
temperature, 2, 29, 36, 38, 47, 59, 68, 79, 90, 91, 94, 128, 174, 175, 186, 187, 190, 194, 195, 196
tempo, 225
tendon, 297, 298, 310, 313, 403
tendons, 250, 297, 298, 310, 313, 356
tensile strength, 301
tension, 310, 313
tenure, 114
territory, 245
testing, 129, 154, 256, 308
testosterone, 303, 360, 406, 407, 424, 426, 431
TGA, 308
TGF, 212, 285, 314, 315, 328, 333, 334, 341, 342, 343, 344, 349, 350, 357, 363, 364, 365, 410, 411, 418
Thailand, 35, 54
therapeutic agents, 288
therapeutic approaches, 435
therapy, 272, 275, 285, 301, 303, 306, 327, 341, 348, 353, 382, 394, 395, 404, 413, 419, 434
thermogravimetric analysis, 308
thinking, 266
thorax, 300
threat, xi, 35, 45, 46, 128, 182
threatened, 174
threonine, 344
thrombin, 286, 290, 292
thrombosis, 273, 282, 286, 289, 290, 294, 295, 338, 349
thrombus, 282, 286, 289, 349
thymus, 332, 339, 340
thyroid, xiv, 157, 158, 161, 162, 163, 168, 314, 315, 365, 397

thyroid cancer, 397
thyroid gland, 158
thyroxin, 365
tibia, 101, 103, 107, 109, 111, 112, 113, 114, 142, 229, 327
tides, 10
tiger, 152, 154
timing, 19, 251, 252
tissue homeostasis, 280, 403, 431
titanium, 322, 323
tobacco, 225, 232, 235
tolerance, 158, 162, 163
tonic, 309
topology, 120, 121
total product, xiv, 185
toxic, 174, 179
toxic effect, 179, 424
toxicity, x, 28, 29, 35, 36, 37, 43, 46, 47, 139, 174, 182, 183, 375, 433
toxicology, 36, 54
toxin, x, 27, 179, 182
tracking, 166
trade, 128
training, 310, 373, 409
traits, 247
transaminases, 163
transcription, 58, 62, 64, 66, 73, 163, 208, 211, 219, 227, 230, 234, 236, 237, 245, 246, 247, 253, 260, 261, 263, 264, 265, 266, 268, 270, 307, 363, 403, 420
transcription factors, 211, 227, 237, 263, 268, 363
transcripts, 61, 62, 212, 228, 237, 242, 250, 258
transduction, 86, 246, 277, 317, 344, 350, 377
transection, 287, 297, 338, 402
transfection, 384
transformation, 5, 166, 167, 232, 363, 364, 386, 396, 397, 417
transformations, 166
transforming growth factor, 314, 363, 364
transfusion, 398
transgene, 249, 284
transition, 284, 374, 429
translation, 119, 237, 246, 253, 255
translocation, 267, 320, 363
transmembrane glycoprotein, 320
transmission, xv, 205, 206, 207, 215, 217, 228, 264, 354, 413, 415, 417
transmission electron microscopy, 228, 264, 354, 413, 418
transplantation, 232, 274, 285, 334, 400
transport, 58, 191, 219, 246, 265
transverse section, 269
trauma, 166, 169

trees, xiii, 18, 117, 118, 119, 120, 121, 122, 123
 tribes, 114
 trifluoroacetic acid, 197
 triggers, 84, 213, 248, 249, 344, 345
 triiodothyronine, 158, 365
 trochanter, 101, 142
 tropism, 209
 trout, 138, 139, 140
 tumor, 167, 169, 217, 235, 276, 280, 282, 306, 332, 353, 358, 361, 374, 375, 376, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 393, 394, 395, 396, 397, 401, 422, 427
 tumor cells, 167, 276, 280, 374, 375, 378, 380, 382, 386, 389, 391, 397, 401
 tumor growth, 306, 374, 389, 394
 tumor invasion, 361, 375, 397
 tumor metastasis, 282, 388
 tumor necrosis factor, 217, 332
 tumor progression, 280, 353, 375, 383, 388, 390, 397
 tumorigenesis, 377, 380
 tumors, 167, 169, 170, 281, 305, 306, 375, 377, 392, 393, 394, 397, 423
 tumours, 371
 turbulence, 90
 turnover, 2, 11, 13, 82, 84, 163, 306, 391
 type 1 collagen, 410
 tyrosine, 277, 325, 344
 tyrosine hydroxylase, 325

U

ultrastructure, 85, 228
 ultraviolet, 167
 umbilical cord, 400
 UNESCO, 28, 46
 uniform, x, 29, 30, 37, 38, 47, 103, 133, 210
 United States, 239, 374
 urbanization, 206
 urethra, 359
 urinary bladder, 305, 306, 307
 urine, 221
 uterus, 83, 85, 279, 355, 417, 420, 431
 UV, xv, 167, 185, 190, 195, 236
 uveitis, 207

V

vaccine, 140, 217
 vagina, 417
 validation, 246, 260
 valuation, 356
 values, xiii, 127, 129, 131, 132, 133, 136, 137
 variability, 6, 8, 13, 154, 233, 418

variables, 94, 175, 379
 variance, 6, 7, 79, 81, 82, 90
 variations, xii, 9, 11, 67, 72, 73, 83, 87, 94, 134, 170, 317, 322, 348
 vascular endothelial growth factor (VEGF), 353
 vascular wall, 284
 vasculature, 232
 vasculitis, 337
 vector, 206, 216, 234, 237, 252, 260, 384, 389
 vegetables, 18, 366
 vein, 229, 294, 349, 394, 400, 431
 velocity, 310, 368
 Venezuela, 13, 221
 ventricle, 372
 venules, 352
 vertebrates, 128, 228, 233, 237, 243, 250, 256, 284, 318
 vesicle, 262, 426
 vessels, xv, 205, 207, 231, 232, 285, 305, 312, 333, 336, 337, 352, 391, 402, 405, 406, 407, 423, 425, 432
 video, 345
 video microscopy, 345
 villus, 72, 73
 viral infection, 353
 virus, 167
 viruses, 354
 visual system, 255
 visualization, 304
 vitamin D, 73
 VLA, 11, 333, 334, 340
 vulva, 105, 107, 112, 114

W

walking, xii, 30, 47, 99
 warts, 167, 168
 waste, 128, 401
 waste products, 128
 water, 128, 130, 137, 138, 158, 174, 179, 180, 181, 183
 water quality, 183
 wavelengths, 353
 weakness, 308, 309
 weight gain, 163, 307, 417, 434
 weight loss, 207
 wells, 327, 347, 401
 western blot, 277, 342, 344, 363, 364, 383
 wheat, 141, 277
 wheat germ, 277
 wild animals, 151
 wild type, 230, 232, 236, 241, 251, 253, 287, 329, 339, 349, 358

wildlife conservation, xiii, 117
winter, ix, 1, 3, 5, 8, 10
withdrawal, 320
women, 168, 314, 355, 374
wood, 24, 100
workers, 229, 238, 248, 257, 262, 266
World Bank, 221
World Health Organization (WHO), 170
wound healing, 286, 299, 306, 343, 362, 394, 405,
412, 419, 425, 427, 428, 429

X

xenobiotic, 182
xenobiotics, 182, 183

X-ray, 327

Y

Y chromosome, 152, 252
yeast, x, xv, 37, 39, 40, 41, 193, 194, 195, 197, 200,
201, 202, 203, 245, 261, 344
Yemen, 186
yolk, 57, 63, 65, 187, 246, 278

Z

zinc, 139, 361, 391, 392, 435
zoology, 186
zooplankton, ix, xii, 87, 88, 90, 91, 92, 93, 94