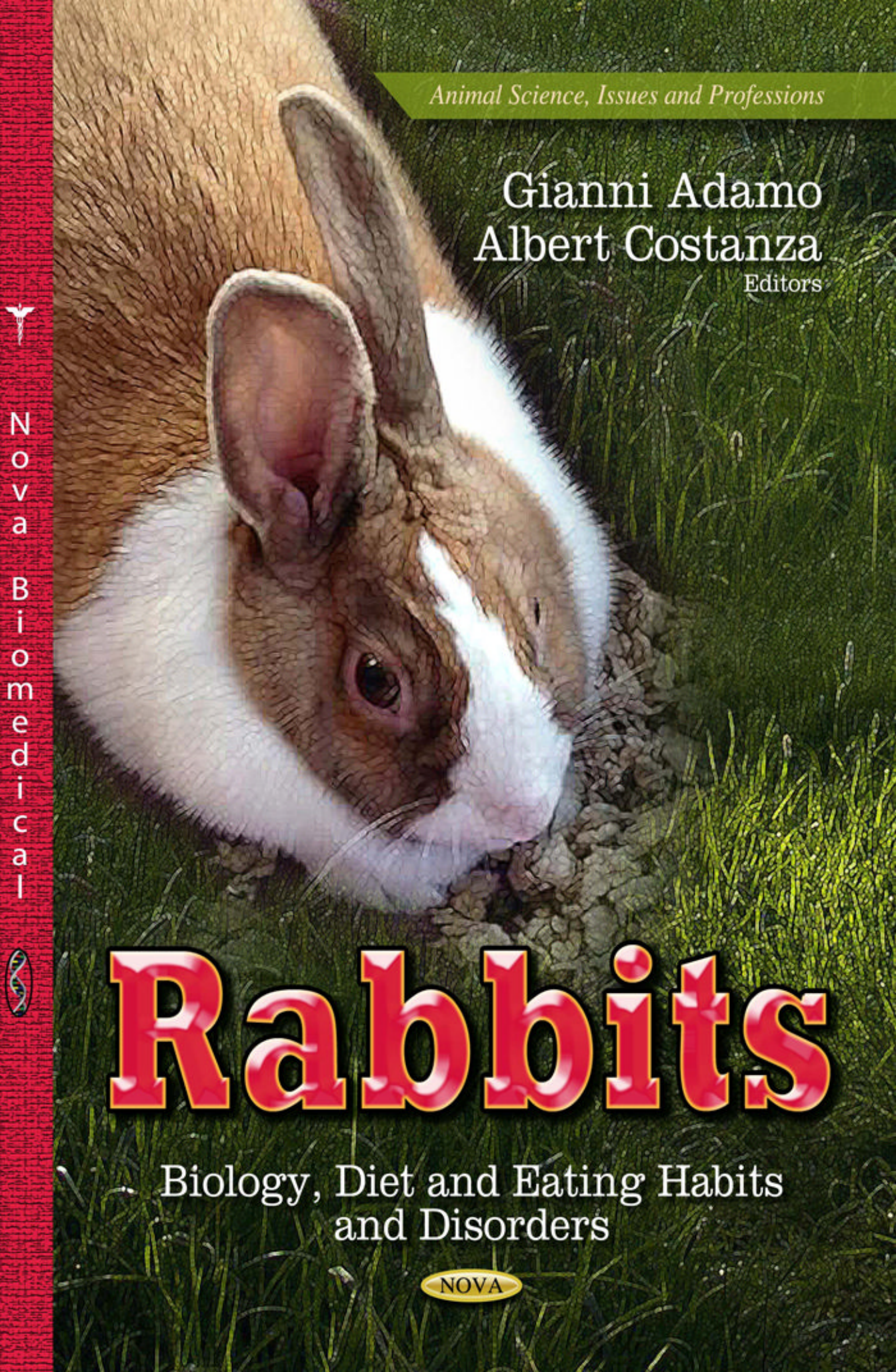

Animal Science, Issues and Professions
Gianni Adamo
Albert Costanza
Editors
Nova Biomedical
Rabbits
Biology, Diet and Eating Habits and Disorders
NOVA

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

RABBITS

BIOLOGY, DIET AND EATING HABITS AND DISORDERS

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

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

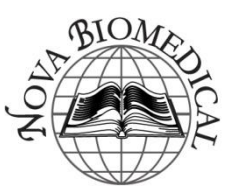
Additional e-books in this series can be found on Nova's website
under the e-book tab.

ANIMAL SCIENCE, ISSUES AND PROFESSIONS

RABBITS

BIOLOGY, DIET AND EATING HABITS AND DISORDERS

GIANNI ADAMO
AND
ALBERT COSTANZA
EDITORS



New York

Library of Congress Cataloging-in-Publication Data

Rabbits : biology, diet and eating habits and disorders / editors, Gianni Adamo and Albert Costanza.
pages cm
Includes index.
ISBN: 978-1-62948-268-2 (eBook)
1. Rabbits. 2. Rabbits--Feeding and feeds. I. Adamo, Gianni, editor of compilation. II. Costanza, Albert, editor of compilation.
SF453.R228 2011
599.32--dc23

2013036361

Published by Nova Science Publishers, Inc. † New York

Contents

Preface

In this book, the authors present current research in the study of the biology, diet, eating habits and disorders of rabbits. Topics discussed in this volume include the rabbit brain as a model of structural neuroplasticity; a survey on the studies of rabbit prion proteins; the effect of reduced dietary consistency on the fiber properties of rabbit jaw muscles; the use of rabbits to investigate the pathogenesis of disease; the assessment of sperm DNA damage in rabbits using the Halomax assay; nutritional effects of plant oil and seeds in rabbit feeding; and current studies on the etiology of obstructive dysfunction of the male rabbit urinary bladder.

Chapter I – Plasticity is the ability to make adaptive changes related to the structure and function of a system. The central nervous system of mammals is endowed with structural plasticity, although prevalently composed of genetically-determined neural circuits which confer it a substantially static structure. Since two decades we know that such plasticity also involves the genesis of new neurons in the postnatal period and during adulthood (adult neurogenesis). Adult neurogenesis in mammals has been investigated mainly in laboratory rodents (mice and rats), and to a lesser extent in other species. Beside some differences concerning the amount of neurogenesis and its possible functional role, the genesis of new neurons in mammals is generally restricted to the same brain regions (neurogenic zones). Unexpectedly, some additional neurogenic processes have been found to occur in different brain regions of postnatal and adult rabbits. Despite the Orders Lagomorpha and Rodentia being quite similar, such difference is quite astonishing. In addition, some features of rabbit neurogenesis are more similar to humans than to rodents. Taking into account that adult neurogenesis is a phylogenetically highly conserved feature, yet with strong adaptation to the ecological niche of each animal groups, the rabbit could be an excellent model for studying the logic of neuroplasticity in mammals and its possible role in brain repair.

Chapter II - In 2012 Nov-Dec, an article named “Naturally prion resistant mammals: a utopia?” seems to have killed all the articles of the past few decades reporting rabbits are resistant to prion infection. As we all well know, “Multiple amino acid residues within the rabbit prion protein inhibit formation of its abnormal isoform”. Which is right and which is wrong? Thus, at this moment, it is very worth doing a detailed survey on the studies of rabbit prion proteins by this article.

Chapter III - The dynamic nature of muscle fibers enables them to adapt to altered functional requirements by changing their myosin heavy chain (MyHC) isoform and cross-sectional area. Although these changes may occur under various conditions, muscular activity plays an essential role in modulating the phenotypic properties of muscle fibers. In jaw muscles, muscular activity is influenced by the consistency of the available food. It has been

shown that the continuous intake of a liquid diet, which eliminates masticatory effort, reduces muscular activity and, in turn, the functional capacity of jaw muscles. However, little is known about how jaw muscles respond to a reduction in dietary consistency within the normal range of compressive strengths of foods. Therefore, the present study investigated the effect of the long-term intake of such a diet on the MyHC composition and the fiber cross-sectional area of jaw muscles in the rabbit.

Male juvenile rabbits were randomly divided into two groups, which were raised on diets of different consistency from weaning to puberty. The experimental group was fed pellets requiring significantly reduced peak loadings, and thus lower level of jaw-muscle activity, to break the pellet in comparison with the standard pellets fed to the control group. At puberty, the MyHC composition and the corresponding cross-sectional area of fibers in the superficial masseter, superficial temporalis, medial and lateral pterygoid, and digastric muscles of both groups were determined using immunohistochemistry.

The proportion and cross-sectional area of fibers co-expressing MyHC-I and MyHC-cardiac alpha were significantly smaller in the masseter muscles of the animals that had been fed the soft pellets than in those of the controls. In contrast, the proportions and cross-sectional areas of the various fiber types in the other jaw muscles did not differ significantly between the groups.

These findings suggest that the long-term reduction in the dietary consistency contributes to selective disuse resulting in structural adaptation of the masseter muscle, the jaw-closing muscle primarily responsible for generating occlusal force during chewing, reflected in decreases in the proportion and cross-sectional area of its slow fibers. At the same time, it appears that the reduced activity during mastication of a diet with the consistency of soft foods normally eaten by rabbits is sufficient to prevent disuse atrophy in the other jaw muscles. Overall, the rabbit masticatory system seems to be relatively rigid, manifesting few diet-specific changes in the fiber properties of jaw muscles.

Chapter IV - The rabbit (Oryctolagus cuniculus) belongs to the taxonomic order Lagomorpha and exists in three forms: wild, feral and domestic. This good natured and medium-sized animal is widely used in biomedicine to investigate many aspects of human disease. Data generated from rabbits have provided researchers with a wealth of useful scientific information. Pharmacological, surgical, immune and genetic investigations have reinforced the notion that they have physiological and anatomical similarities to man, facts that can be exploited in disease prevention and treatment studies.

In recent years the scope of their use has increased dramatically and now covers a myriad of human conditions: cerebrovascular disease including Alzheimer’s, cardiovascular, reproductive including erectile dysfunction, lung, liver, kidney and bone diseases, as well as viral infection. Development of transgenic rabbit models has also opened up new opportunities to look at disease-induced alterations in the genome. This chapter describes these models and evaluates their usefulness in reproducing the clinical characteristics and treatment of various human diseases.

Chapter V - While it may be possible to obtain a broad view of semen quality from the rabbit ejaculate using assessments of sperm concentration, motility and morphology, none of these estimates provide information about the integrity of sperm chromatin, which in other species, has proven to be an interesting new approach for gaining insight about the semen sample with regards to its potential for successful fertilization and embryonic development. This study was conducted to evaluate a sperm DNA fragmentation assessment methodology

based on the capacity of controlled protein depletion (Halomax-assay) to assess rabbit SDF and which was validated against indirect (comet assay) and direct (*in situ* nick-translation) measures of sperm DNA damage. There was a high degree of correlation between estimates of sperm DNA fragmentation using the Halomax and the neutral comet assay (r^2 = 0.964; $p<0.001$); there was also a direct correlation between Halomax assay and the results obtained from DNA labelling using forced polymerase nucleotide incorporation by means of the *in situ* nick translation assay. The validated Halomax assay was then used to determine the baseline level of SDF and sperm DNA longevity from 63 HYLA commercial breed rabbits and calculated as the rate of SDF degradation (r-SDF) following incubation at 38 ºC for 7h. The mean (± SEM) baseline level of SDF obtained after ejaculation was 9.1 ± 1.2 %. The mean (± SEM) rate of SDF (rSDF) obtained after 7h of incubation was 2.62 ± 0.34% per h. Differences in the baseline level of SDF and in the r-SDF were found among different animals. The procedures outlined in this study now allow SDF measurement to be implemented in rabbit AI centres as a routine assay to select for high quality semen doses with reduced levels of chromatin damage after ejaculation and which show a low rate of DNA damage following incubation in an environment that mimics the female reproductive tract.

Chapter VI - This study aims to review various rabbit experiments on the effects of supplementating mixed feed with plant oils (corn and palm) or oilseeds (false flax, golden flax, chia, and perilla seeds). Apparent digestibility and lipid traits in the *longissimus dorsi* muscle of growing rabbits fed with these fat sources are reviewed. Moreover, our results are compared with similar works on rabbit nutrition.

Fat sources influence the fatty acid (FA) composition of rabbit body fat. Even though meat and meat products are often associated with nutrients considered to be unhealthy, in fact rabbit meat offers excellent nutritive and dietetic properties. Further improvements to rabbit meat production can be achieved by adding functional compounds associated with animal feed fortification or enrichment. Among these functional compounds long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) have many known beneficial effects on health.

False flax, golden flax and chia seed supplementation in mixed feed increases the apparent digestibility of most of the nutrients, and false flax and golden flax in particular improved ether extract digestibility in weaned crossbred rabbits. A similar result was also obtained in rabbits fed mixed feed supplemented with corn oil, with less impressive resultsfor those supplemented with palm oil.

The FA profile in rabbit meat mainly reflects the dietary oil sources. The saturated FA and monounsaturated FA contents were lower in the muscles of animals fed the corn oil diet than in those of rabbits given the palm oil diet. In contrast, total PUFA and n-6 PUFA content was lower in the muscles of rabbits fed the unsaturated palm oil diet than in animals given the corn oil diet.

In rabbits fed a diet with increasing quantities of oilseed, the saturated FA and monounsaturated FA proportions in the *longissimus dorsi* muscle decreased, while PUFA increased. Oilseed dietary supplementation has shown to be effective in raising the n-3 PUFA proportion in the meat and decreasing the n-6/n-3 PUFA ratio.

In conclusion, these results showed that the use of diets with corn oil or supplemented with different oilseeds was effective in reducing the saturation, atherogenic and thrombogenic indexes, with consequent benefits on the nutritional quality of rabbit meat for consumers, with no significant adverse effects on digestibility in growing rabbits.

Chapter VII - Bladder dysfunction secondary to benign prostatic hyperplasia (BPH) is a major affliction of aging men. Although the symptoms of BPH are related to the effects of an enlarging prostate there appears to be no direct relationship between prostate size and severity of obstructive bladder dysfunction. Therefore, urodynamic findings cannot accurately predict either level of bladder pathology or potential for recovery following surgery or pharmacological therapy. Thus, biomarkers that can identify the severity of male obstructive bladder dysfunction and at what point the dysfunction becomes irreversible would be of significant value in the management of the disorder.

The progression of obstructive bladder dysfunction from compensated bladder function through severe bladder decompensation is mediated primarily by four processes: 1) Cyclical ischemia followed by reperfusion (I/R) mediated by the hypertrophied bladder smooth muscle and compression of the blood vessels during contraction. 2) Increase in intracellular free calcium mediated by the ischemia resulting in activation of specific intracellular proteases, lipases, and phospholipases that result in cellular and intracellular damage. 3) The progressive increase in the severity of the I/R results in increased free radical generation that cannot be handled by the intracellular antioxidant mechanisms such as superoxide dismutase (SOD) and catalase resulting in progressive obstructive bladder dysfunction. And 4) progressive replacement of functional smooth muscle with connective tissue mediated by the cellular and subcellular damage to the muscle resulting in irreversible full stage obstructive dysfunction.

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter I

The Rabbit Brain As a Model of Structural Neuroplasticity

Luca Bonfanti[1,4,*], Federico Luzzati [2,4], Roberta Parolisi[1,4], Paola Crociara [1,4] and Giovanna Ponti [3,4]

[1]Department of Veterinary Sciences
[2]Department of Life Sciences and Systems Biology
[3]Department of Neuroscience
[4]Neuroscience Institute Cavalieri-Ottolenghi (NICO),
Orbassano (TO), University of Turin, Italy

Abstract

Plasticity is the ability to make adaptive changes related to the structure and function of a system. The central nervous system of mammals is endowed with structural plasticity, although prevalently composed of genetically-determined neural circuits which confer it a substantially static structure. Since two decades we know that such plasticity also involves the genesis of new neurons in the postnatal period and during adulthood (adult neurogenesis). Adult neurogenesis in mammals has been investigated mainly in laboratory rodents (mice and rats), and to a lesser extent in other species. Beside some differences concerning the amount of neurogenesis and its possible functional role, the genesis of new neurons in mammals is generally restricted to the same brain regions (neurogenic zones). Unexpectedly, some additional neurogenic processes have been found to occur in different brain regions of postnatal and adult rabbits. Despite the Orders Lagomorpha and Rodentia being quite similar, such difference is quite astonishing. In addition, some features of rabbit neurogenesis are more similar to humans than to rodents. Taking into account that adult neurogenesis is a phylogenetically highly conserved feature, yet with strong adaptation to the ecological niche of each animal groups, the rabbit could be an excellent model for studying the logic of neuroplasticity in mammals and its possible role in brain repair.

* Correspondence: Luca Bonfanti DVM, PhD. Department of Veterinary Sciences, and Neuroscience Institute Cavalieri Ottolenghi (NICO), Regione Gonzole, 10 - 10043 Orbassano (TO); Tel. 0039 011 6706606; Fax 0039 011 6706621; E-mail: luca.bonfanti@unito.it.

Introduction

Plasticity, namely the ability to make adaptive changes related to the structure and function of the system, is an essential attribute of biological tissues and organs allowing adaptation to challenging environment and compensatory adaptation after traumatic injury or pathology. In addition, at least in some tissues, plasticity can also produce repair and regeneration (Ferguson and O'Kane, 2004). Nevertheless, such reparative capacity remarkably vary in different tissues/organs. Those undergoing continuous cell renewal (e.g., skin, blood, cornea, many epithelia) do contain multiple and disperse units of stem cell niches (e.g., intestinal crypts, hair follicle bulge in the skin, endosteal and perivascular hematopoietic niches in bone marrow; Nystul and Spradling, 2006; Morrison and Spradling, 2008). After injury, regeneration in these 'labile' tissues is favored by the persistence of undamaged stem cell niches. On the other hand, the adult central nervous system (CNS) of mammals can be considered as substantially static, both under the profile of cell renewal and of tissue repair (Bonfanti, 2011, 2013). The CNS reparative capacity is higher in invertebrates and non-mammalian vertebrates, whereas it has been largely lost in some animal groups, such as birds and mammals (Masaki and Ide, 2007; Bonfanti, 2011). A fundamental feature of CNS structure is its connectional, neurochemical and functional specificity which allows specific cell types to be connected and to act in a relatively invariant way (Frotscher, 1992). The mammalian CNS is a highly complex structure made up of a huge number of neurons (10^{11}), an even higher number of glial cells (10^{12}) and around 10^9 synapses per cubic millimetre ($10^{15}/mm^3$ in humans; Chklovskii et al., 2004). Its architectural specificity is attained during development and maintained in the adult through a vast cohort of membrane-bound and extracellular matrix molecules, mainly involving adhesion molecules and their receptors with permissive and/or instructive functions (Gumbiner, 1996). Most of these molecules, as well as different types of inhibitory factors, are also responsible for CNS stabilization thus leading to the well known incapability of mammals to undergo neuronal regeneration and repair (Caroni, 1997). In addition, with respect to other organs, the CNS shows structural peculiarities since neurons and glial cells possess ramified, intermingled processes, some of which projecting very far from the cell body. This fact makes the CNS not simply a ‘mass’ of organized cells but a complex set of circuits in which a remarkable portion is composed of ‘cables’ and synaptic contacts. For this reason, along with the old allocation of nerve cells as ‘perennial’ (non-renewable), the word «regeneration» in neuroscience was originally restricted to the re-growth/re-elongation of cell processes by surviving cell bodies after injury (e.g., axonal regeneration (Davies et al., 1997).

Besides this feature of substantially static architecture, *structural plasticity* is another attribute of the CNS, complementary to specificity (Zilles, 1992).

CNS Structural Plasticity

In addition to molecular changes (functional adaptive plasticity), which theoretically does not affect the shape of cells, structural plasticity involves changes which modify the shape and structure of the CNS at the cellular level (Bonfanti, 2006; Theodosis et al., 2008). An important distinction should be made between developmental and adult structural plasticity,

the former, being at the basis of CNS formation. Developmental plasticity is fundamental for building up the specificity of the mature CNS, allowing the occurrence of morphogenetic processes which involve massive structural changes (e.g., cell proliferation, cell migration, axonal/dendritic growth), undergoing a progressive downregulation during the postnatal period (Bonfanti and Peretto, 2011). The transition from developing to mature CNS leads to a stabilization of connections and ultimately to a substantially unrenewable tissue. The time-course of such a transition depends on the species, usually being linked to the complexity of the brain and its functions (Kornack, 2000). What remains after the downregulation of developmental plasticity is adult plasticity, which can be considered as an exception to the rule, restricted to specific locations, at the anatomical and/or the cellular level (Bonfanti, 2006, 2011, 2013).

In the extraordinary heterogeneity of CNS cellular composition different aspects of plasticity frequently overlap. Hence, the concept of structural plasticity can be further dissected by considering different types of cells (glial, neuronal) and different parts of the cell involved, as well as their relationships with the surrounding elements. Progressive degrees of morphological changes can be found: from synaptic plasticity, to axonal/dendritic growth and neuron-glial plasticity. A profound change in the concept of structural plasticity occurred in the 1990s, starting from two striking findings: the occurrence of a adult neurogenesis and cell migration within the forebrain subventricular zone (SVZ; Lois and Alvarez-Buylla, 1994), and the first isolation of adult neural stem cells (Reynolds and Weiss, 1992). These studies strengthened the idea that most typical developmental processes, ultimately converging to the genesis of new neurons, actually exist in the adult mammalian CNS (Gage, 2000; Alvarez-Buylla and Garcìa-Verdugo, 2002; Kempermann, 2002; Carleton et al., 2003). Yet, neural stem cells of the mammalian CNS are restricted to two stem cell niches (neurogenic zones; see below). In many renewable tissues, the ability to dynamically redistribute and activate new niches is an important strategy for regenerative capacity (Wright et al., 2001; Morrison and Spradling, 2008). By contrast, in some stable organs showing low cell renewal rates, but retaining remarkable potentialities for compensatory hyperplasia upon functional demands, e.g., kidney and liver, stem cells and their niches appear to be less active (Kung and Forbes, 2009; Little and Bertram, 2009). As to the CNS, although we know that it is remarkably plastic under the profile of neural connection reorganization, e.g., experience-dependent (structural) synaptic plasticity (Holtmaat and Svoboda, 2009), the high topographical restriction of the only two stem cell niches do not allow regeneration in most of the neural parenchyma (Bonfanti, 2011,2013).

Neurogenesis

The word 'neurogenesis' indicates all those processes leading to building up the CNS through embryonic and fetal phases. Final CNS architecture is the result of cell divisions and precise cell-cell and cell-substrate interactions starting from a few undifferentiated cells in the neural tube (Bayer and Altman, 2004). The primitive neural tube is organized around the ventricular cavities which partially will persist in the adult filled with cerebrospinal fluid. In the neurogenic process, peri-ventricular germinative layers provide the source for most neuronal and glial cell precursors. These precursors subsequently populate the CNS

parenchyma mainly by centrifugal, radial migration from the neuraxis toward the pial surface, following a precise spatial and temporal pattern which allows undifferentiated cells to differentiate in their final destination and to establish appropriate connections (Rakic, 1990; Misson et al., 1991; Marin and Rubenstein, 2003). The primitive germinal layers consist of a ventricular zone (VZ) containing direct descendants of the primitive neuroectoderm (neuroepithelium), and a subventricular zone (SVZ), which later emerges from the VZ. The SVZ contains rapidly proliferating cells and expands at early postnatal development, eventually giving rise to subsets of neurons and glial cells (Levison, 2006).

Hence, the embryonic CNS is a highly dynamic environment undergoing dramatic changes in cell number and position, paralleled and followed by refinement of cell relationships at single projection and synapse level. After birth, through a brief postnatal period whose length depends both on species and brain complexity/size (Kornack, 2000), the CNS parenchyma undergoes stabilization, thus making plasticity an exception to the rule.

Adult Neurogenesis

Twenty years ago, the discovery was made that neural stem cells do exist even in the adult CNS (Reynolds and Weiss, 1992) accounting for adult neurogenesis throughout life within restricted brain regions of many mammals (Lois and Alvarez-Buylla, 1994; Gage, 2000; Kornack and Rakic, 2001a; Bernier et al., 2002; Luzzati et al., 2003; Rodriguez-Perez et al., 2003; Amrein et al., 2007), including humans (Eriksson et al., 1998; Sanai et al., 2004; Curtis et al., 2007). New neurons are continuously produced within two neurogenic sites harbouring neural stem cell niches, the forebrain subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) (reviewed in Bonfanti and Ponti, 2008). Since then, a lot of detail has been made available about the cell composition, structure, organization, function and modulation of these neurogenic regions (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Bonfanti and Theodosis, 1994; Doetsch et al., 1997; Gage, 2000; Alvarez-Buylla and Garcìa-Verdugo, 2002; Carleton et al., 2003; Kempermann et al., 2004; Seri et al., 2004; Zhao et al., 2006; Ihrie et al., 2011).

Forebrain SVZ

In laboratory rodents, an anterior extension of the SVZ forms a solid chord of migrating cells through the olfactory peduncle and bulb axes called *rostral migratory stream* (RMS; Lois and Alvarez-Buylla, 1994; Bonfanti and Theodosis, 1994; Figure 1). Both SVZ and RMS contain two main cell compartments: i) newly generated, migrating neuroblasts, in the form of tangentially-oriented *chains* (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996), and ii) protoplasmic astrocytes organized to form a dense meshwork throughout the SVZ area, giving rise to longitudinally-oriented channels called *glial tubes* (Lois et al., 1996; Peretto et al., 1997; Figure 1).

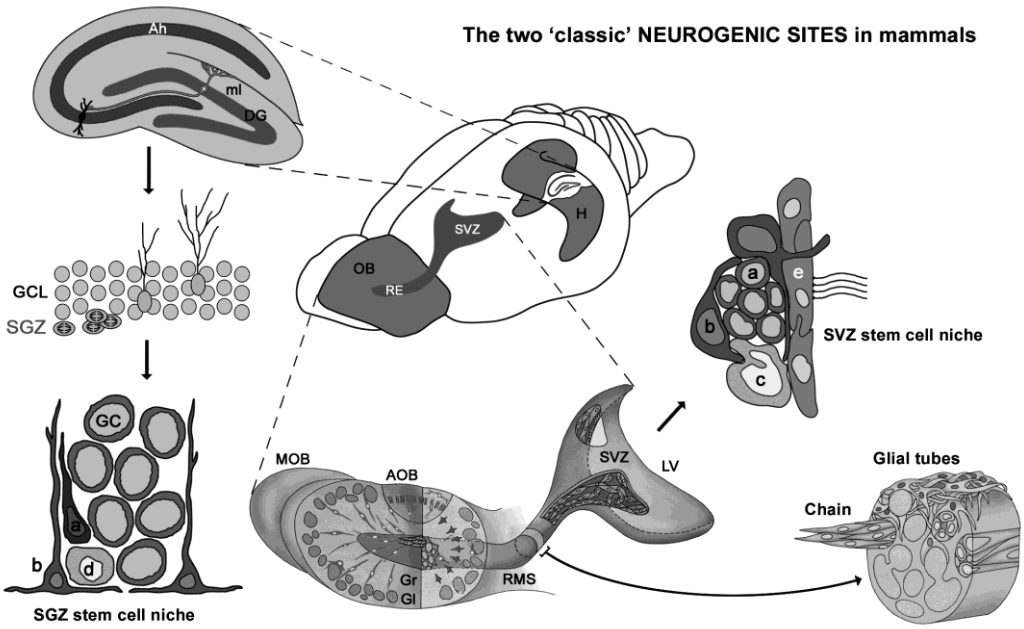


Figure 1. Schematic representation of the two main neurogenic sites in the adult rodent brain (adapted from Bonfanti and Ponti, 2008). Genesis of new neurons occurs in two main brain areas (in the middle): the olfactory bulb (OB) and the hippocampus (H). Their germinative layers are respectively the SVZ (bottom and right) and the subgranular zone (SGZ, left). The SVZ is composed of two main compartments: astrocytic *glial tubes*, and tangential *chains* of neuronal precursors, the latter sliding within the glial ensheathment (adapted from Peretto et al., 1997). In electron microscopy they correspond to type A cells (a) and type B cells (b). A third cell type (C cell, c) is present in the stem cell niche (left, top; adapted from Doetsch et al., 1997); e, ependyma. The SVZ chains dissolve in the olfactory bulb, whereby single neuroblasts move radially through the main (MOB) and accessory (AOB) olfactory bulb layers (Gr, granular layer; GL, glomerular layer). RE, rostral extension (rostral migratory stream). The hippocampal SGZ is located in the inner layer of the dentate gyrus (DG, left top) and gives rise to granule cells (GC) with dendrite arborisation in the molecular layer (ml) and an axon projecting to the Ammon's horn (Ah). Even in the absence of glial tubes and chains, cell types similar to those described in the SVZ form a stem cell niche in the SGZ (left, bottom; adapted from Seri et al., 2004). For details on the functional relationships among different cell types, see text.

The SVZ was the first site in which neural stem cells were isolated (Reynolds and Weiss, 1992) and identified as astrocytes (Doetsch et al., 1999), being considered as the major stem cell reservoir of the mature brain (Gage, 2000). Neuroblasts (also referred to as type A cells; Lois et al., 1996) have a leading and trailing process, a large nucleus and a thin rim of electrondense cytoplasm, with abundant free ribosomes. Astrocytes (type B cells) are stellate-shaped cells detectable by immunocytochemical staining for GFAP and deriving from postnatal transformation of embryonic radial glia (Merkle et al., 2004). In addition to A and B cell types, a third element with intermediate ultrastructural features and high proliferative capacity has been identified as type C cells, considered as 'transit amplifying' cells forming a bridge between slow proliferating stem cells and their progeny (Doetsch et al., 1997; Ponti et al., 2013). After a few cell divisions occurring within 4 days, each astrocytic neural stem cell is estimated to give rise to 16 young neurons (neuroblasts; Ponti et al., 2013). Neuroblasts generated in the rodent SVZ migrate within the glial tubes, performing a particular type of

PSA-NCAM-dependent tangential 'chain migration' which allows a long distance displacement (about 5 mm in mice) towards the olfactory bulb (Bonfanti and Theodosis, 1994; Lois and Alvarez-Buylla, 1994; Hu et al., 1996). Chain migration is proper to adult neurogenesis, the first SVZ chains being detectable during the second/third postnatal week in rodents (Peretto et al., 2005). Once in the olfactory bulb, the migrating neuronal precursors leave the glial tubes and pursue their migration radially as isolated neuroblasts through parenchymal layers of the main (Lois and Alvarez-Buylla, 1994) accessory olfactory bulb (Bonfanti et al., 1997; Figure 1). Most of these cells differentiate within the granular layer as GABA-containing granule cells, whereas a smaller subpopulation reaches the glomerular layer becoming dopaminergic periglomerular cells (Betarbet et al., 1996; De Marchis et al., 2004). By marking newly born granule cells with GFP-encoding retrovirus injected in the SVZ, it has been shown that both granule (Carleton et al., 2003) and periglomerular neurons (Belluzzi et al., 2003) grow dendritic spines, display spontaneous synaptic currents, and form synapses with other bulbar neurons, although they have distinct electrophysiological properties from old neurons. Thus, the SVZ provides new local inhibitory interneurons that synaptically integrate into olfactory bulb circuitry likely regulating the spatial and temporal coding of mitral and tufted cells at the first sensory relay of olfaction. It has been estimated that about 1% of total olfactory bulb interneurons are added each day in the adult, one-half of them dying between 15 and 45 days after their birth (Petreanu and Alvarez-Buylla, 2002; Doetsch and Hen, 2005).

Hippocampal SGZ

The dentate gyrus is part of the hippocampal formation, located in the dorsal-caudal part of the telencephalon, beneath the corpus callosum (Witter and Amaral, 2004). It is a three-layered cortex (allocortex) made up of small neurons called granule cells which form a 4-10 cell thick layer comprised between two fiber layers (the hilus and the molecular layer). Progenitor cells divide in the inner part (SGZ), with the progeny giving rise to mature granule cells that extend an axon within the mossy fibre pathway during the first four days which reaches Ammon's horn during the first month (Hastings and Gould, 1999; van Praag et al., 2002; Zhao et al., 2006; Figure 1). Most newly generated cells die during the first two weeks (Kempermann et al., 2003). The surviving elements translocate their cell body through the outer layers for a few microns and successfully integrate into circuits in about six weeks (Hastings and Gould, 1999; Van Praag et al., 2002). It has been estimated that about 250.000 new neurons are added to the rodent dentate gyrus within one month, representing 6% of the total number in this area (Cameron and McKay, 2001). The generation of hippocampal cells in humans was recently analysed by measuring the concentration of nuclearbomb-test-derived ^{14}C in genomic DNA, estimating a rate of 700 new neurons per day (Spalding et al., 2013). The rate of adult hippocampal neurogenesis significantly decreases with age, more evidently than the forebrain SVZ (Kuhn et al., 1996). Since hippocampal newly born cells differentiate locally, no chain migration can be found in the dentate gyrus. Instead of SVZ glial tubes, radial and horizontal astrocytes are present, the former being a special type of SGZ radial glia-like astrocyte, similar to the type B cells described in the forebrain (Figure 1). These glial cells can divide and give rise to the granule cell precursors, probably representing the stem

cell compartment of the hippocampus (Seri et al., 2004). Hippocampal neurogenesis is modulated by many factors. It has been used as a model for quantitative studies aimed at unravelling how cell production and survival are regulated. For example, an enriched environment increases the survival of newly born cells (Kempermann et al., 1997; Gould et al., 1999a) whereas stress conditions exert the opposite effect (Mirescu and Gould, 2006). Hormonal levels (Tanapat et al., 1999), physical activity (Van Praag et al., 1999) and diverse experimental/lesion paradigms (Parent et al., 1997; Kokaia and Lindvall, 2003) also affect hippocampal neurogenesis, although the exact mechanisms are still obscure (Gould et al., 1999a; Mirescu and Gould, 2006).

Notwithstanding differences in the anatomical distribution and cytoarchitecture of the two neurogenic sites, a common pattern can be found in their cell composition under a morphological and functional profile. Both the SVZ and the SGZ are persistent, active germinative layers associated respectively with the anterior part of the lateral ventricles and the inner layer of the hippocampal dentate gyrus (Figure 1). Astrocytic stem cells in both neurogenic sites do derive from VZ radial glia cells (Kriegstein and Alvarez-Buylla, 2009). While the SVZ maintains direct contact with the ventricles, the SGZ loses such contact during development. The SVZ neuronal precursors undergo long-distance migration to reach their final site of destination in the olfactory bulb whereas those generated within the dentate gyrus differentiate locally. Once integrated, these cells prevalently replace dead neurons in the olfactory bulb or add to preexisting populations in the hippocampus (Imayoshi et al., 2008). In both systems, adult neurogenesis is modulated during learning of different tasks, thus it may play a role in learning and memory (Gould et al., 1999a; Leuner et al., 2006; Lledo et al., 2006). From the functional point of view, adult neurogenesis has been related to adaptation to ecological pressures (Barker et al., 2011). At present, this is one of the most satisfactory functional explanations in the entire phylogenetic tree, along with multiple, genetically determined variables spanning from the brain anatomy/developmental history to the animal lifespan (Amrein et al., 2011). This range of possibilities can also be increased by non-genetic variables, such as experience-dependent cues (Johnson et al., 2010; Barker et al., 2011; Kempermann, 2012). Benefits of adult neurogenesis appear to converge on increased neuronal and structural plasticity subserving coding of novel, complex, and fine-grained information, usually with contextual components that include spatial positioning (Konefal et al., 2013). Three main hypotheses for the functions and adaptive significance of adult neurogenesis, which are not mutually exclusive, involve pattern separation, memory consolidation, and olfactory spatial. Roles for neurogenic plasticity have also been suggested for a wide range of human physiological functions and pathological conditions (Sohur et al., 2006; Konefal et al., 2013).

A link between Adult Neurogenesis and Brain Repair Is not Granted

At the beginning of the nineties, the discovery of neural stem cells and adult neurogenesis led many people to consider definitively broken the dogma of the CNS as made up of non-renewable elements (Gage, 2000; Gross, 2000). This finding triggered new hopes for brain repair, yet, twenty years later, the dream of regenerative medicine applied to CNS injuries and

neurodegenerative diseases is still very far (Arenas, 2010; Lindvall and Kokaia, 2010). As a matter of fact, adult neurogenesis in mammals occurs mainly within the two restricted neurogenic sites (see above), and, as a direct consequence of such topographical localization, most of the CNS parenchyma out of the SVZ and SGZ remains substantially a non-renewable tissue (Bonfanti, 2013). An indirect proof of this statement resides in the fact that most of the traumatic/vascular injuries and neurodegenerative diseases, which actually occur in "non-neurogenic" regions, have still not found efficacious therapies capable of restoring CNS structure and functions through cell replacement (Arenas, 2010).

Nevertheless, during the last decade, new heterogeneity has been revealed by studies showing a substantial and widespread gliogenic (Horner et al., 2000; Dawson et al., 2003; Butt et al., 2005; Nishiyama et al., 2009), and to a lesser extent, neurogenic potential (Dayer et al., 2005; Luzzati et al., 2006; Ponti et al., 2008) within the CNS parenchyma, namely, in those areas previously considered as non-neurogenic (reviewed in Bonfanti and Peretto, 2011; Bonfanti, 2013). This new field of investigation revealed many unexpected potentialities for *de novo* cell genesis in the CNS, although most aspects of parenchymal neuro-glio-genesis remain quite obscure and ill-defined.

Parenchymal Neurogenesis

All neurogenic processes occurring outside the two germinal layers (SVZ and SGZ) are indicated as *parenchymal* neurogenesis, since located in the CNS parenchyma. Spontaneous parenchymal neurogenesis can be considered as a very rare phenomenon in mammals, and its regional location has been shown to be dependent on the animal species, age, and physiological/pathological states (Bonfanti and Peretto, 2011). Different examples have been described in rodents (Dayer et al., 2005; Kokoeva et al., 2005), rabbits (Luzzati et al., 2006; Ponti et al., 2008), and monkeys (Gould et al., 2001; Bernier et al., 2002), with remarkable interspecies differences. Most parenchymal neurogenesis described in adult rodents seems to occur spontaneously at very low levels, rather being elicited/enhanced after specific physiological or pathological conditions (Ohira et al., 2009; Pierce and Xu, 2010).

Among the unsolved issues of parenchymal neurogenesis are the numerous reports which have not been confirmed by further studies or by other laboratories (Gould et al., 1999b; Magavi et al., 2000; Nakatomi et al., 2002; Zhao et al., 2003; Rivers et al., 2008; Guo et al., 2010), along with a series of data which have been denied in studies trying to reproduce the same results (Kornack and Rakic, 2001b; Richardson et al., 2011; Frielingsdorf et al., 2004). Hence, it is evident that we still not grasp the real limits and/or opportunities of parenchymal neurogenesis and that further studies are required before finally accepting, or denying the existence of each 'unusual' neurogenic process. On the other hand, what appears clear is that some stem/progenitor cells in the parenchyma are able to give rise to new neurons in experimental and/or pathological conditions (Luzzati et al., 2011; Ohira et al., 2009; Pierce and Xu, 2010). Various examples of 'reactive' neurogenesis are known to occur after different types of CNS injury. Beside neurogenesis induced from adjacent neurogenic sites (Arvidsson et al., 2002; Thored et al., 2006), some neurogenic/gliogenic processes are also thought to start from local, parenchymal progenitors (Luzzati et al., 2011; Ohira et al., 2009; Komitova et al., 2006). For instance, local progenitors in layer I of the rat cerebral cortex, which in

normal conditions seem to be rather quiescent, are activated after ischemia giving rise to new cortical interneurons (Ohira et al., 2009). Also in a slow and progressive model of striatal neuronal degeneration, besides activation of SVZ progenitors, genesis of neuroblasts has been found to occur also from local progenitors in mice (Luzzati et al., 2011). This suggests that certain pathological states can stimulate either migration of progenitors from the adult SVZ or activation of local neuronal progenitors. Yet, one of the issues which remain poorly investigated is whether the adult brain parenchyma belonging to spontaneously non neurogenic areas could be endowed with quiescent progenitor cells which can be stimulated to awake under specific environmental conditions, independently from the contribution of germinal layers.

A case placed in between the spontaneous and experimentally-induced neurogenesis is that of the hypothalamus. Several publications based on experiments carried out on rodents have been reporting data on this brain region as a new site for adult constitutive neurogenesis in mammals (for review see Cheng, 2013).

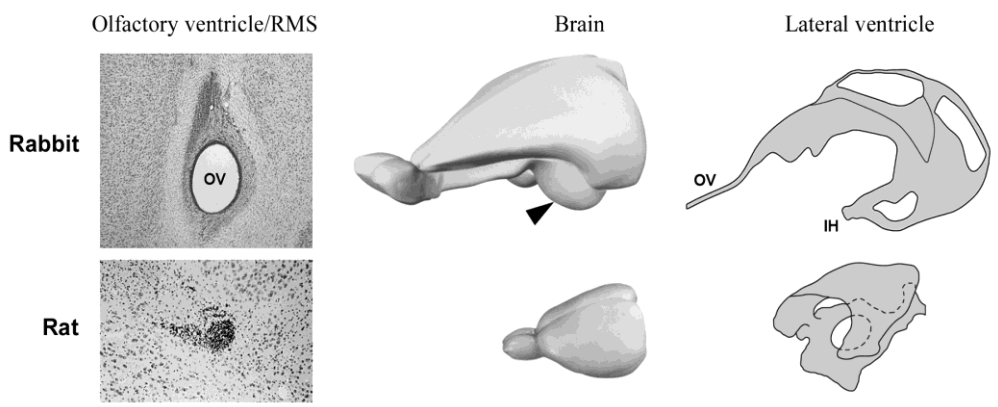


Figure 2. Differences in the overall organization of forebrain ventricular cavities in rabbit and rodents: an open olfactory ventricle (OV) remains associated with the SVZ rostral extension (rostral migratory stream, RMS) of rabbits; another extension of the lateral ventricle, the inferior horn (IH) is present in the well developed temporal lobe of lagomorphs (arrowhead). These ventricular extensions are absent in the smaller brain of rodents (adapted from McFarland et al., 1969; Leonhardt, 1972; Bonfanti and Ponti, 2008).

Several unresolved aspects make parenchymal neurogenesis a difficult territory to be explored: (i) the contrast between a wide range of potentialities displayed by parenchymal progenitors isolated *in vitro* and far more restricted potentialities which can be observed *in vivo* (Palmer et al., 1999; Belachew et al., 2003), (ii) the existence of studies reporting neurogenesis in parenchymal regions which have been denied or not confirmed by other researchers (Gould et al., 1999b; Magavi et al., 2000; Kornack and Rakic, 2001b), and (iii) the real origin of progenitors which are induced to proliferate/migrate in different lesion models (either mobilized from neurogenic sites or activated locally within the parenchyma (Arvidsson et al., 2002; Nakatomi et al., 2002; Thored et al., 2006; Luzzati et al., 2011).

In conclusion, alternative and multiple forms of plasticity involving neurons can overlap within the CNS parenchyma, affecting preexisting cells/circuits and increasing the complexity of the whole picture of brain structural remodeling. Notably, in recent years differences in

adult neurogenesis have been emerging among mammals, so that there are several reasons for further analysis of adult mammalian neurogenesis in a comparative perspective.

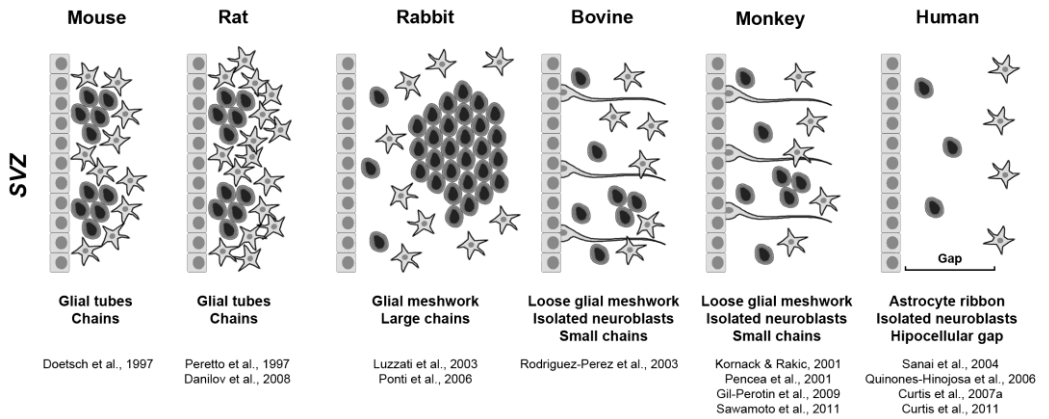


Figure 3. Organization of the subventricular zone (SVZ) neurogenic niche in different mammals. Most differences concern the astrocytic spatial organization and the neuroblast assembly into chains. Type C cells and displaced ependymal cells are not showed. Although still fragmentary, the data available suggest some general principles: (i) chain assembly could be linked to robust and fast cell migration; (ii) chain assembly and cell migration are not directly linked with persistence/closure of the olfactory ventricle; (iii) glial meshwork density is not directly linked with chain assembly nor cell migration. Note that rabbit SVZ architecture is different from both that of rodents and that of animals endowed with large brains (bovine, monkey, humans), yet more similar to that of humans in the part located close to the ventricle (adapted from Bonfanti and Peretto, 2011).

The Rabbit as a Model for Postnatal and Adult Neurogenesis

The New Zealand white rabbit (*Orictolagus cuniculus*) belongs to the lagomorphs (order Lagomorpha), forming a distinct order compared with rodents (order Rodentia), despite being grouped together in the past. Rabbits are both domestic and laboratory animals, yet from the neurological point of view they are mainly used as models in vision research (Manning et al., 1994).

As neuroanatomy is concerned, in contrast with a deep knowledge in rodents few detailed studies have been performed in lagomorphs, making it difficult to set up comparative considerations. Two evident features concerning rabbit brain anatomy can be relevant to adult neurogenesis: the occurrence of more prominent cerebral ventricular extensions and more expanded temporal lobes (Figure 2). Differences in the overall brain anatomy have been proposed as relating to a different shape and extension of the ventricles, since "cerebral ventricles are primordial hollows of the brain that reflect in changes of their configurations and size the development and growth of the neural tube" (McFarland et al., 1969). Besides a basic common plan, the ventricles of mammals vary in relation to the different degrees of expansion of different CNS parts (e.g. the neocortex can vary up to 10.000-fold range in surface in different species; Kornack, 2000).

The lateral Ventricles and SVZ of Rodents and Lagomorphs

In many domestic animals including dogs, cats, cows, sheep and rabbits the lateral ventricle is prolonged into a persistent *olfactory ventricle*, whose walls do not collapse after birth (McFarland et al., 1969; Leonhardt, 1972; Figure 2). This feature does not seem to be related to brain size since an open olfactory ventricle is absent and replaced by a solid chord of tissue both in rodents and primates, including humans (McFarland et al., 1969). The tissue forming the rostral migratory stream in rodents is replaced in rabbits by a SVZ rostral extension ensheathing the dorsal part of the olfactory ventricle (Figure 2). Another extension of the lateral ventricular system called *inferior horn* and protruding ventrally within the temporal lobe, is absent in rodents but present in rabbits and primates, and correlates well to the expanded temporal lobes of these species (McFarland et al., 1969; Leonhardt, 1972; Figure 2). This trend is confirmed by the occurrence of a *posterior horn* in species with massive development of the occipital lobes and high degree of visual specialization, maximally evident in primates and humans.

The rabbit SVZ, delineated as an area in which a high proliferation rate, chains of PSA-NCAM+/doublecortin+ cells, and an astrocytic meshwork do overlap (Luzzati et al., 2003; Ponti et al., 2006a), extends along the lateral wall of the lateral ventricle and the dorsal part of the olfactory ventricle (rostral extension). From the posterior part of the lateral ventricle, a ventral lateral extension (Luzzati et al., 2003) lines the lateral ventricle inferior horn, somehow adapting to a different ventricular conformation with structures which are absent in rodents.

Cytoarchitecture of the Rabbit SVZ

Concerning the rabbit SVZ internal arrangement, three main differences are found compared with rodents: (1) heterogeneous distribution of the chain and glia compartments; (2) simultaneous occurrence of isolated neuroblasts and very large chains; (3) existence of a looser astrocytic meshwork (Ponti et al., 2006a). Two distinct parts can be detected all along its extension: a ‘ventricular’ SVZ, adjacent to the ventricular wall and containing small aggregates of neuroblasts immersed within a relatively dense glial/ependymal sheath, and an ‘abventricular’ SVZ, detached from the ventricles and containing large chains of neuroblasts immersed in a loose network of astrocytic processes (Figure 3). This distinction is reinforced by a thick band of tissue, poor in cells and enriched in nerve fibres and glial processes, which could be reminiscent of the ‘hypocellular gap’ of the human SVZ (Quinones-Hinojosa et al., 2006; Figure 3). Another common feature is the absence of well organized glial tubes, replaced by an incomplete astrocyte row reminiscent of the human ‘astrocyte ribbon’ (Sanai et al., 2004). Something similar has also been described in the bovine SVZ (Rodriguez-Perez et al., 2003; Figure 3), suggesting that in non-rodent mammals the glial meshwork could be less tightly-packed and less compartmentalized than in rodents. On the other hand, a striking difference between rabbit and human SVZ consists of large chains in the rabbit abventricular SVZ in contrast with the absence of chains on both sides of the astrocyte ribbon in humans. About 10-20 medium-large chains are visible, some of which include up to 15-20 nucleated cells in transverse section (Ponti et al., 2006a; schematically represented in Figure 3). Thus, the SVZ appears structurally more heterogeneous in lagomorphs than in rodents, suggesting

that both chain and glia compartments can differ in their architecture and mutual relationship, in relatively close mammalian species. Studies carried out on rodents show SVZ chains are not present at birth but they assemble around the third postnatal week (Peretto et al., 2005). In rabbits the pattern of chain formation is observed very early, starting from postnatal day 10 (Ponti et al., 2006a). This difference is strengthened by the fact that postnatal development is temporally different in these species, being earlier in rodents (puberty occurs in the first or second postnatal months) than in rabbits (around the fourth month). On the other hand, the rabbit SVZ glial compartment will never attain the degree of organization typical of rodent glial tubes (Peretto et al., 1997, 2005), thus appearing to retain a certain degree of 'morphological immaturity' for quite a long period. After ultrastructural analysis of the SVZ associated with the lateral ventricle, in which most of the stem cell niche is expected to occur, the same cell types described in rodents were found, yet with a different spatial organization. The absence of glial tubes and chains in the rabbit ventricular SVZ, along with the occurrence of astrocytes forming a row parallel to the ependyma, makes this structure somehow more similar to the human stem cell niche than that of rodents (Doetsch et al., 1997; Sanai et al., 2004; Quinones-Hinojosa et al., 2006; summarized in Figure 3).

Extensions of the Rabbit SVZ within the Brain Parenchyma

In addition to its internal arrangement, another intriguing feature of the rabbit SVZ is the occurrence of numerous extensions entering the surrounding mature parenchyma (Figure 4). These *parenchymal chains* were identified after serial reconstruction of brain tissue sections immunostained for PSA-NCAM revealing two groups of chain-like aggregates: (1) anterior chains, leaving the rostral extension and immersed within the corpus callosum beneath the frontal cortex (Figure 4), and (2) posterior chains, apparently leaving the ventral-lateral extension, along the external capsule (Luzzati et al., 2003). The real nature of chains is confirmed by ultrastructural reconstruction and analysis of BrdU-treated animals at different post-injection survival times, showing no cell proliferation within the parenchymal chains yet revealing the occurrence of newly generated cells after 5-10 days (Luzzati et al., 2003; Ponti et al., 2006a). The immunoreactivity for PSA-NCAM and doublecortin on these tangentially-oriented bulks of cells as well as the ultrastructural features of bipolar neuroblasts displayed by their cellular components are both features of 'chain migration' (Lois et al., 1996; Luzzati et al., 2003; Ponti et al., 2006a). On the other hand they are relatively glia-independent, being directly in contact with axons (anterior chains) or at the interface between white and gray matter, including contact with mature neurons and oligodendrocytes (posterior chains). Similarly to abventricular SVZ chains, parenchymal chains are very large, abundantly trespassing the average number of cells found in rodents (usually 3-5). The 'compact' morphology of anterior chains (Figure 4) is consistently different from the more irregular, 'laminar' arrangement of the posterior ones, made up of clusters and rows of cells which partially disaggregate and re-aggregate as they progress through the tissue. Anterior chains show continuity with large chains of the abventricular SVZ, in some cases following blood vessels in their shift from SVZ to white matter (Ponti et al., 2006a). The affinity between chains of neuroblasts and blood vessels appears quite important since it has been demonstrated to occur in the mouse striatum following a stroke (Yamashita et al., 2006). Interestingly, the occurrence of rabbit anterior chains is limited to the postnatal/peripuberal

period (Ponti et al., 2006a). This is another feature similar to humans, in which a stream reaching the ventro-medial prefrontal cortex has been described during the first 18 months of life (Sanai et al., 2011; see also Bonfanti and Peretto, 2012).

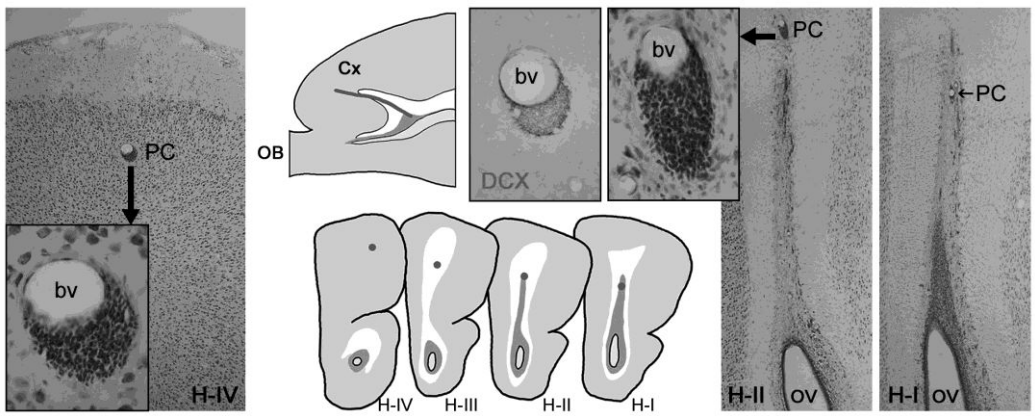


Figure 4. An anterior parenchymal chain of neuroblasts (PC) in a 6-month-old rabbit. After reconstruction, the chain is followed along a blood vessel (bv) through the white matter of the corpus callosum and in the frontal cortex (Cx). On the right another parenchymal chain viewed with electron microscopy within the corpus callosum. ov, Olfactory ventricle; DCX, doublecortin (from Bonfanti and Peretto, 2012).

On the other hand, the posterior parenchymal chains due to their fragmentary arrangement and distribution could be made up of locally generated elements characterized by inconstant relationships with the SVZ. Indeed, even appearing as a homologue of the 'temporal stream' described in monkeys (Bernier et al., 2002) they could hardly be considered as a true SVZ extension. The in vivo observations carried out on rabbits show that chains of neuroblasts can be found in various contexts but suggest that cell migration outside the SVZ and its glial meshwork, although possible, could turn out more difficult and inefficient by the occurrence of non-glial substrates. In other terms, if a glial substrate is not essential for the occurrence of chain migration, when it does occur in the form of sheaths (glial tubes) it could provide a favourable environment.

Notwithstanding the occurrence of many PSA-NCAM+ cells with typical bipolar-shaped morphology of migrating elements, radially-oriented in the cortical/subcortical areas adjacent to parenchymal chains, no newly generated cells are detectable in the cortex and a few newly born cells were occasionally observed in the amygdala one month after the BrdU treatment (Luzzati et al., 2003).

In conclusion, migration in parenchymal chains could be discontinuous and slow if compared to that directed to the olfactory bulb through the SVZ. Although it is very unlikely that such alternative routes could provide functional cell addition/renewal in the brain parenchyma (apart from the amygdaloid regions), their existence show the heterogeneity of SVZ arrangement in mammals leaving open possibilities for modulation of such endogenous sources of cell progenitors in the perspective of brain repair.

Protracted Neurogenesis in the Cerebellum

Mammalian neurogenesis has a species-specific timetable, with detectable differences at both embryonic and postnatal stages. This is related to differences in length of gestation and postnatal growth period prior to puberty (Bayer and Altman, 2004). In addition to species-specific variations, the temporal windows of onset and downregulation of neurogenetic processes can be heterogeneous throughout the neuraxis. For instance, most neocortical neurons are generated during mid-gestation whereas some neuronal cell populations continue to be added after birth in the cerebellum, hippocampus and olfactory bulb (reviewed in Rakic et al., 2004). Thus, although in most CNS regions germinative layers are exhausted at birth, in some locations they persist postnatally or throughout adulthood (Figure 5).

The mammalian cerebellum is a typical example of postnatal neurogenesis aimed at establishing a huge population of granule cells during the period in which the animal is interacting with the external environment. For such 'neurogenesis regionalization', the cerebellum is a remarkable model of *protracted neurogenesis* (Bonfanti and Peretto, 2011).

Although the genesis of most cerebellar cell types occurs very early from the periventricular neuroepithelium lining the 4th ventricle (Figure 6 A), interneurons and some astrocytic glial cells complete their centrifugal migration through the white matter and their specification postnatally (Maricich and Herrup, 1999; Grimaldi et al., 2009). Cell proliferation of these progenitors still occurs in prospective white matter. In addition, the postnatal mammalian cerebellum undergoes a protracted genesis of granule cells through a transitory, secondary germinative layer localized on its surface: the external germinal layer (EGL; Figure 6 A-C).

The EGL is formed by tangential subpial displacement of cell precursors from the germinal trigone of the 4th ventricle, then leads to protracted genesis of the granule cell population by radial, centripetal migration of cell precursors. This transitory germinal zone progressively reduces its thickness as the granule cell precursors migrate deep into the cortex, then disappear at specific ages in different species (from 3 weeks in mice to 11 months in humans, which is very early compared with puberty; reviewed in Ponti et al., 2008, 2010; Figure 6 A-C).

In rodents, the delayed proliferation, specification and differentiation of glial cells and interneurons coming from the prospective white matter is concluded before the end of granule cell genesis (Grimaldi et al., 2009; Leto et al., 2009). After this stage, no more cell genesis is detectable, as no germinal layers remain active, so that cerebellar plasticity throughout life is granted solely by synaptic changes in pre-existing circuits. Under the functional profile, this delayed genesis of granule cells and interneurons shares a logic with the role of cerebellar circuits in learning / adapting motor skills to the environmental cues the animal is dealing with during postnatal / young stages of its life. This process does not involve simply the addition of new neurons, but also the choice between different types of cell specification (Grimaldi et al., 2009; Leto et al., 2009). The delayed cerebellar neurogenesis might be considered as part of the critical periods that allow formation of new synaptic contacts as well as involving the recruitment of new neurons.

Recent comparative work carried out by our laboratory on the cerebellum of New Zealand white rabbits revealed a far more complex situation, as in these lagomorphs protracted neurogenesis extends around and beyond puberty (Ponti et al., 2006b, 2008).

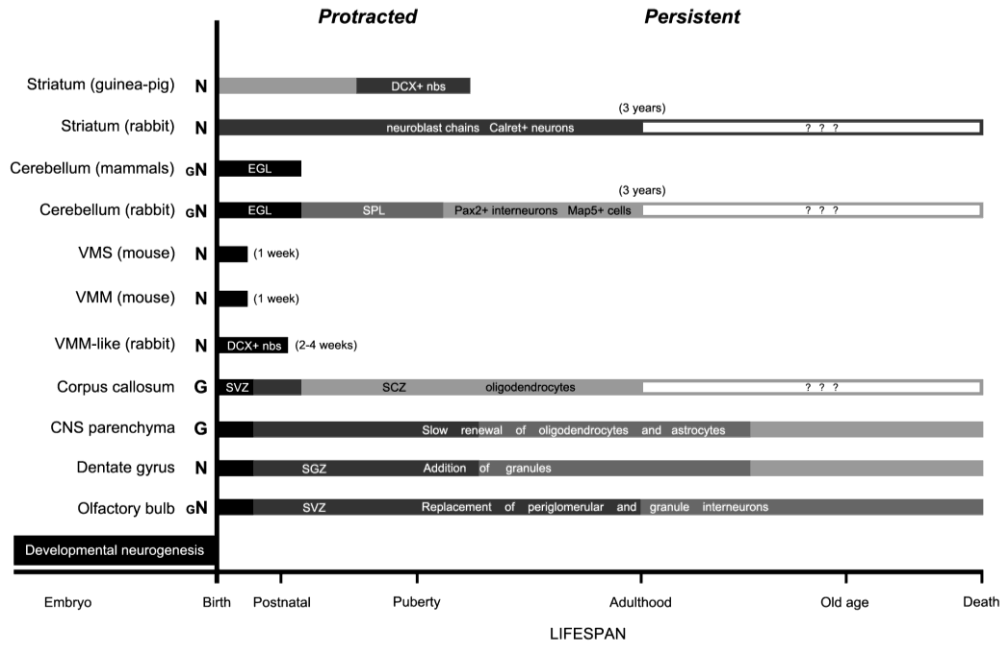


Figure 5. Different developmental extensions of protracted and persistent neurogenesis. Black, postnatal extensions of embryonic neurogenesis (delayed morphogenesis). Shades of grey indicate different rates of cell genesis, usually decreasing with increasing age (note the exception of the guinea pig). DCX, doublecortin; EGL, external granule layer; G, glial progeny; gN, glial and neuronal progeny (prevalently neuronal); Map5, microtubule-associated protein 1B; N, neuronal progeny; nbs, Neuroblasts; SCZ, subcallosal zone; SGZ, subgranular zone; SPL, subpial layer; SVZ, subventricular zone; VMM, ventral migratory mass; VMS, ventral migratory stream (adapted from Bonfanti and Peretto, 2011).

The Rabbit Subpial Layer (SPL)

Unlike the forebrain, the mammalian cerebellum was considered incapable of any spontaneous cell production after the delayed genesis of granule cells occurring in the postnatal cerebellar cortex (see above). In rodents, after EGL exhaustion direct contact between pia mater/Bergmann glial endfeet and parallel fibres characterizes the cerebellar surface (Altman and Bayer, 1997) and no signs of cell proliferation can be detected in the cerebellar cortex (Bonfanti et al., 1992; Dusart et al., 1999; Ponti et al., 2006b). These facts match with the notion of the cerebellum as made up of non-renewable elements. Nevertheless, we reported in rabbits the existence of a secondary germinal matrix persisting beyond puberty in subpial position, called *subpial layer* (SPL, Ponti et al., 2006b), thus suggesting that this is not a general rule in mammals.

The SPL originates from structural modification of the EGL and is capable of generating PSA-NCAM+/doublecortin+ neuronal precursors oriented tangentially on the cerebellar surface and assembled into chains (Figure 6 C,D). Thousands of subpial chains are regularly arranged with a medial to lateral orientation and cover the whole cerebellum from the 2nd to the 5th month of life, then disappearing after puberty. We showed that subpial chains are made up of neuronal precursors and share features with forebrain SVZ chains (Ponti et al., 2006b). Cell proliferation occurs among chains, the newly born neuroblasts being incorporated within them in the subsequent days. By studying the shift from EGL to SPL in young rabbits we

found that SPL chains form through fragmentation of the EGL pre-migratory layer around the end of the first postnatal month, then increase their distance and progressively dilute on the cerebellar surface until they disappear (Figure 6 C,D,G). Although we do not have direct evidence of cell migration in rabbit SPL chains, they could be involved in the tangential displacement of neuronal precursors which has been shown to occur in the mouse EGL pre-migratory layer prior to engagement in radial migration (Komuro et al., 2001). The lengthy persistence of rabbit SPL chains do suggest that remarkable structural plasticity could persist in the relatively mature environment of the peripubertal rabbit cerebellar cortex. The newly generated cells of the SPL are detectable far beyond the estimated end of rabbit granule cell genesis (Smith, 1963) and show an absolutely different morphology and distribution compared with the 'ectopic' granule cells described in the molecular layer of the rabbit cerebellar cortex (Spacek et al., 1973).

Interestingly, in parallel with the persistence of the SPL, high amounts of PSA+/doublecortin+ newly born cells were also detectable within the cerebellar cortex, and some of them were still present at subsequent ages, after the disappearance of the SPL. An hypothesis explaining the occurrence of young cortical cerebellar neurons in the absence of an active germinative layer could be linked to a delayed arrival of immature interneuron precursors coming via radial migration from the underlying white matter, as classically described for cerebellar cortex interneurons of neuroepithelial origin (Maricich et al., 2001).

Genesis of Interneurons in the peripuberal and Adult Cerebellar Cortex

Until a few years ago, it was universally accepted that in the cerebellum all processes of delayed neuronal cell genesis were exhausted with the end of granule cell genesis and concurrent EGL (Altman, 1972) or SPL (Ponti et al., 2006b) disappearance. Yet, further studies carried out on rabbits revealed substantial genesis of cerebellar interneurons until peripubertal ages, and to a lesser extent, in adult animals (Ponti et al., 2008). These cells are PSA-NCAM+/doublecortin+/Pax2+ neurons that continue to proliferate within the cerebellar cortex parenchyma in the absence of any residual germinal layers (Ponti et al., 2008, 2010). The presence of the trancription factor Pax2 indicate that these cells are neuroepithelial-derived elements (Weisheit et al., 2006), thus originally coming from the periventricular germinative layer through withe matter radial migration (see Maricich et al., 2001). This neurogenic process occurs spontaneously in the intact rabbit CNS, even in fully adult animals (1-2 year old rabbits; summarized in Figure 6 G). The newly generated Pax2+ interneurons were followed by BrdU injection and long-term survival (2 months); they also express PSA-NCAM and doublecortin during the first 3 weeks of their life, thus revealing their morphology of bipolar, migratory cells, which is followed by a typical neuronal morphology, being GABA positive, and negative for Sox2 and Olig2 (Ponti et al., 2008; Figure 6 E). We know the cellular source, but it is not clear if cell divisions occur either within the white matter or the cerebellar cortical grey matter (the first BrdU+ / PSA-NCAM+ cells are detectable several days after injection; Ponti et al., 2008), although the occurrence of bipolar double-stained cells suggests they could come from the white matter.

At present, this is the only case of adult neurogenesis known in the mammalian cerebellum. Nevertheless, such a process has been documented in adult rabbits up to 3 years old, then progressively decreasing in intensity (10/1 in the first 6 months, and once again 10/1 from 6 months to 3 years; Ponti et al., 2008).

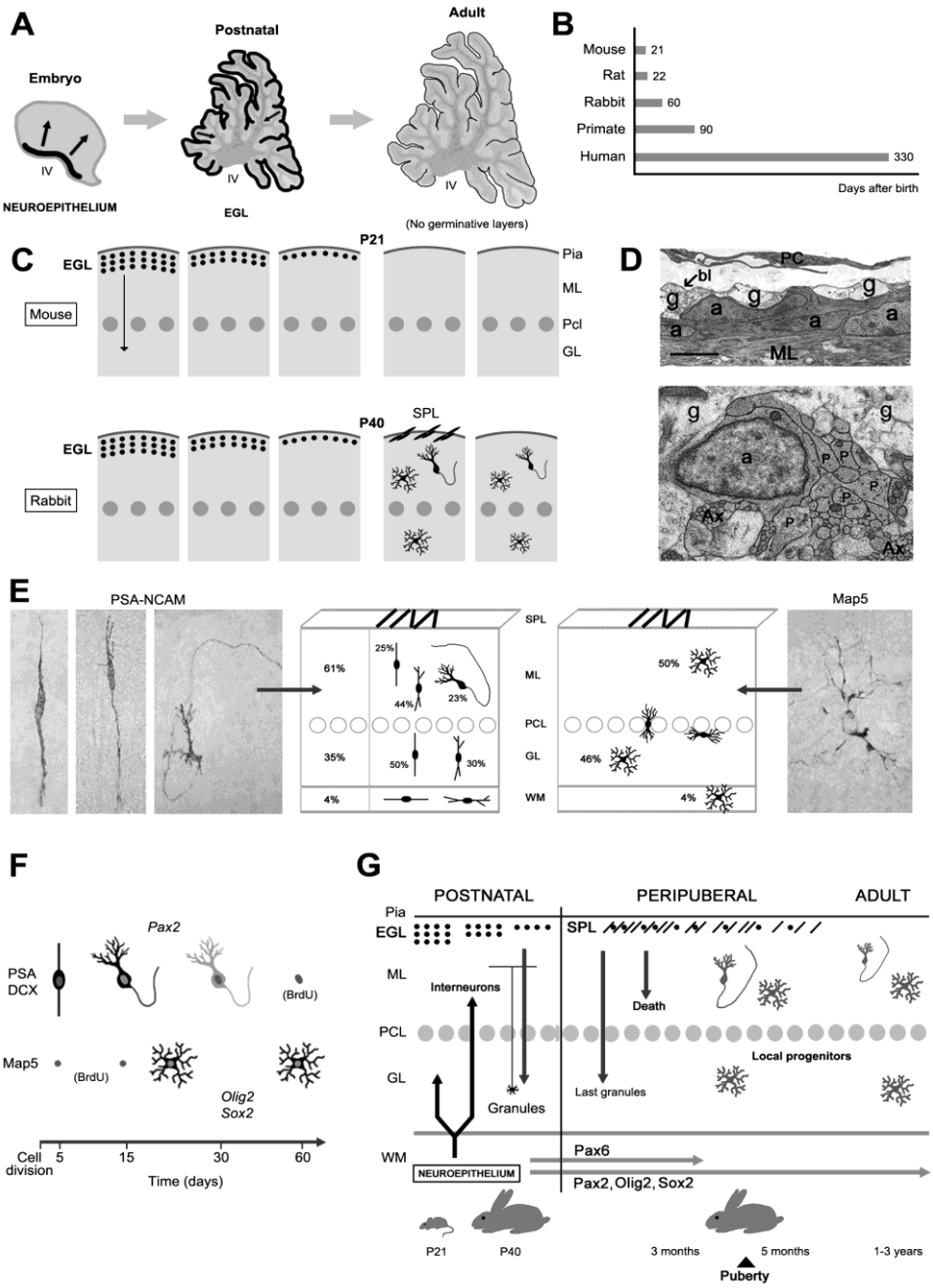


Figure 6. Protracted neurogenesis in the cerebellum of young/adult rabbits. A, Exhaustion of germinative layers in the pre- and post-natal cerebellum of rodents. B, Estimated end of granule cell genesis in different mammals. C, Persistence of subpial layer (SPL) and parenchymal genesis of neuronal and glial precursors in the rabbit cerebellar cortex, with respect to rodents. D, Ultrastrucural evidence for SPL chains of neuroblasts (top, longitudinal; bottom, transversal). E, Two populations of newly generated cells in the peri-puberal rabbit cerebellum (left, neuronal-shaped cells; right, multipolar cells). F, The two cell populations acquire and lose cellular markers at different stages of their postmitotic life. G, Time course of germinative layer-dependent and independent cell genesis in the rabbit cerebellum (adapted from Ponti et al., 2010).

Subsequent ages have not been investigated, yet the substantial decrease in newly generated cerebellar neurons suggests a further dilution/exhaustion of the process in older rabbits.

Under the comparative profile, the rabbit cerebellar neurogenesis appears as a unique example of protracted neurogenesis in mammals whose functional meaning remains obscure. Its decreasing rate suggests that the rabbit cerebellum pursue its growth (and structural plasticity) for extended peripuberal-young/adult periods.

These results obtained in lagomorphs show that remarkable differences may exist in mammals, perhaps requiring further comparative re-examination of postnatal cerebellar development by taking into account different functional aspects. Little is known about which factors determine the timing of the onset of walking, which represents a fundamental milestone in motor development of mammals (altricial vs. precocial mammals; Sanchez-Villagra and Sultan, 2001). Hoofed animals start walking within hours after birth, both rodents and small carnivores require days or weeks, and non-human primates take months (approximately 1 year in humans) to achieve this locomotor skill (Garwicz et al., 2009).

Striatal Adult Neurogenesis

Although different degrees of cell proliferation have been described in the adult striatum of some mammals (see above), full demonstration that the genesis of new neurons can occur spontaneously in this brain region has been provided in lagomorphs. Clusters of newly generated cells do exist in the nucleus caudatus of the rabbit (Luzzati et al., 2006). These cells co-express PSA-NCAM and doublecortin, and form small chain-like structures which have been shown to be independent from the adjacent SVZ by the use of intraventricular cell tracer injections and cultured explants (Luzzati et al., 2006). This spontaneous genesis is attributable to clusters of proliferating cells located within the striatal parenchyma. As in other adult neurogenic sites, PSA-NCAM is transiently expressed on striatal neuronal precursors, being detectable shortly after cell proliferation and disappearing in the late phases of neuronal differentiation into calretinin+ interneurons, herein occurring within 2 months (Luzzati et al., 2006).

Unlike other examples of structural plasticity in rabbit, whose existence is limited to a delayed postnatal period (e.g. anterior parenchymal chains and see below), the occurrence of spontaneous, local striatal neurogenesis has been consistently observed in fully adult animals. Nevertheless, the survival of newly born striatal neurons after 2 months is limited to 1% of the initial BrdU+ cell population, thus indicating the rabbit striatum as a favourable environment for genesis rather than for survival of newly born cells.

Gliogenesis

Another intriguing aspect of plasticity in the adult CNS tissue involves parenchymal glial progenitors which are still capable of cell division during adulthood (reviewed in Dawson et al., 2003; Nishiyama et al., 2009; Boda and Buffo, 2010; Bonfanti, 2013). Most of these progenitors display neural developmental markers of the glial lineage, express a chondroitin sulfate proteoglycan (Nerve/glial antigen 2, Ng2; referred to as Ng2+ cells; Stallcup, 1981),

and are committed to the oligodendrocyte lineage (Nishiyama et al., 2009). The Ng2+ cells are generally considered as synantocytes (Butt et al., 2005) or polydendrocytes (Nishiyama, 2007), endowed with multiple functions in physiology and pathology which are still far from being utterly elucidated. A proportion of these cells persist in the adult in a phenotypically immature form (Dawson et al., 2003; Nishiyama, 2007; Trotter et al., 2010), most of which do continue to proliferate throughout life, thus being considered the main cycling population of the mature mammalian CNS (Simon et al., 2011).

The widespread location of these progenitor cells within the white and grey matter of all brain and spinal cord regions makes them extremely promising as an endogenous source for repair. Yet, in spite of intense investigation carried out during the last decade, such progenitors remain largely obscure in their identity and physiology, due to a scarce availability of stage-specific markers. In particular, what appears difficult is the distinction between real cell populations and various differentiation stages of the same population. We recently focused on a subset of multipolar, polydendrocyte-like cells we found in the rabbit cerebellum (Ponti et al., 2008; Crociara et al., 2013; Figure 6 E). We called these elements mMap5 cells since they express the microtubule associated protein 5 (Map5), which is known to be present in most neurons (Schoenfeld et al., 1989; Riederer, 2007). These cells show a morphology (ramified, multipolar) and a molecular signature (e.g., Olig2 expression) reminiscent of synantocytes/polydendrocytes, and some of them are newly generated within the mature cerebellar parenchyma (Ponti et al., 2008). The Map5 molecule (Riederer et al., 1986; also referred to as Map-1B, Map1X, or Map1.2), belongs to a family of large and fibrous microtubule associated proteins (Maps) and shows a very wide range of expression in the CNS. Map5 is the first Map detectable in neurons of the developing nervous system, expressed at high levels in growing axons/growth cones and usually downregulated after cessation of axonal growth (reviewed in Riederer, 2007). Nevertheless, the protein remains expressed in the whole CNS during adulthood, its phosphorylated form reaching high levels within some regions endowed with plasticity, or under conditions that elicit axonal/synaptic plasticity in relation to physiological conditions and in response to injury (Riederer, 2007). We characterized the morphology, phenotype, regional distribution, proliferative dynamics, and stage-specific marker expression of these cells in the rabbit and mouse CNS (Crociara et al., 2013).

We showed that mMap5 cells can be better visualized in rabbit than in mouse, what probably accounts for the fact they were not described in vivo for long time. In mice, mMap5 cells were never found to co-express the Ng2 antigen. They appear to be a population of glial cells sharing features but also differences with Ng2+ progenitor cells. We showed that mMap5 cells are newly generated, postmitotic parenchymal elements of the oligodendroglial lineage, thus being a stage-specific population of polydendrocytes (Crociara et al., 2013). Interestingly, the number of mMap5 cells is progressively reduced in the brain of adult/old animals, but can increase in neurodegenerative and traumatic conditions (Crociara et al., 2013).

The occurrence of mMap5 cells was also assessed in other mammalian species, including guinea pig, cat, sheep, monkey and human.

Conclusion

In this chapter, some intriguing processes of structural plasticity specifically existing in rabbits have been reported and compared with our knowledge in other mammals. On the whole, the rabbit brain is characterized by the following features: i) the existence of local, parenchymal neurogenesis within the striatum throughout life; ii) the persistence of streams of newly generated neuroblasts reaching the frontal cortex during the juvenile period; iii) the persistence of a transitory germinal layer (subpial layer, SPL) on the cerebellar surface, extending the post-natal EGL beyond puberty; iv) the occurrence of post-puberal and young/adult genesis of interneurons within the cerebellar cortex; v) the presence of an SVZ architecturally different from that of rodents and somehow similar to that of humans.

Most of these processes are examples of 'protracted' neurogenesis, thus extending postnatally embryonic neurogenesis (see Figure 5). Rather than a singular event that suddenly appears during adulthood, adult neurogenesis has long been recognized as the continuation of postnatal neurogenic activity. During the first postnatal weeks, significant cellular changes occur within the germinal layer-derived neurogenic sites: SVZ and dentate gyrus (see for example Tramontin et al., 2003; Peretto et al., 2005) and cerebellar granule cells are generated by the postnatal EGL. Yet, the dynamics of neurogenic processes in the peripuberal and adult rabbit cerebellum are strikingly different from those described in rodents and other mammalian species studied so far (Ponti et al., 2006a, 2008). The production of new cell progenitors, including neuronal precursors, does not cease after the end of granule cell genesis, but continues at remarkable rates up to and beyond puberty, although progressively decreasing with age. Thus, the rabbit cerebellar cortex could represent a permissive environment for widespread parenchymal neurogenesis, as the frontal cortex seems to represent for the anterior parenchymal chains. In mammals, the first postnatal weeks are critical as the brain growth rate is maximal, and changes during this period can have a great impact on overall brain function later in life. In parallel, clinically relevant dysregulations can occur during this postnatal period, and such changes can have an impact on cognitive function later in life (Kuhn and Blomgren, 2001). For its peculiar features in terms of protracted neurogenesi the rabbit could be a good experimental model for studying the neurodevelopmental aspects of adult neurogenesis.

In addition, local, parenchymal cell progenitors capable of generating neurons are present within the rabbit striatum througout life (Luzzati et al., 2006), indicating that in lagomorphs constitutive parenchymal neurogenesis can persist in different CNS regions. In that perspective, some regions of the rabbit CNS could be considered atypical in mammals. What it is not yet clear, at present, is whether an ‘atypical’ niche is required for the activation of resident (quiescent) parenchymal progenitors. Other than investigating the quality of stem/progenitor cells in the perspective of cell transplant or induced local activation, maybe should be also important to understand more about the local tissue environment in which they live.

References

Altman J. Postnatal development of the cerebellar cortex in the rat - The external germinal layer and the transitional molecular layer. *J. Comp. Neurol*., 1972, 145, 353-398.

Altman J., Bayer S.A. (Eds.), Development of the cerebellar system. 1997, CRC Press, Boca Raton, USA.

Alvarez-Buylla A., Garcìa-Verdugo J.M. Neurogenesis in adult subventricular zone. *J. Neurosci*., 2002, 22, 629-634.

Amrein I., Dechmann D.K., Winter Y., Lipp H.P. Absent or low rate of adult neurogenesis in the hippocampus of bats (Chiroptera). *PLoS ONE*, 2007, 2, e455.

Amrein I., Isler K., Lipp H.P. Comparing adult hippocampal neurogenesis in mammalian species and orders: influence of chronological age and life history stage. *Eur. J. Neurosci.*, 2011, 34, 978-987.

Arenas E. Towards stem cell replacement therapies for Parkinson's disease. *Bioch. Biophys. Res. Comm.*, 396, 2010, 152-156.

Arvidsson A., Collin T., Kirik D., Kokaia Z., Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med*., 2002, 8, 963-970.

Barker J.M., Boonstra R., Wojtowicz J.M. From pattern to purpose: how comparative studies contribute to understanding the function of adult neurogenesis. *Eur. J. Neurosci*., 2011, 34, 963-977.

Bayer S.A., Altman J. Development of the telencephalon: neural stem cells, neurogenesis, and neuronal migration. In: Paxinos, G. (Ed.), The Rat Nervous System, 3rd Edition. Academic Press, San Diego, USA, 2004, pp. 27-73.

Belachew S., Chittajallu R., Aguirre A.A., Yuan X., Kirby M., Anderson S., Gallo V. PostnatalNG2 proteoglycan-expressing progenitor cells are intrinsicallymultipotent and generate functional neurons. *J. Cell Biol*., 2003, 161, 169-186.

Belluzzi O., Benedusi M., Ackman J., LoTurco J.J. Electrophysiological differentiation of new neurons in the olfactory bulb. *J. Neurosci*., 2003, 23, 10411-10418.

Bernier P.J., Bédard A., Vinet J., Lévesque M., Parent A. Newly generated neurons in the amygdala and adjoing cortex of adult primates. *Proc. Natl. Acad. Sci. U.S.A.*, 2002, 99, 11464-11469.

Betarbet R., Zigova T., Bakay R.A., Luskin M.B. Dopaminergic and GABAergic interneurons of the olfactory bulb are derived from the neonatal subventricular zone. *Int. J. Dev. Neurosci*., 1996, 14, 921-930.

Boda E., Buffo A. Glial cells in non-germinal territories: insights into their stem/progenitor properties in the intact and injured nervous tissue. *Archiv. Ital. Biol.*, 2010, 148, 119-136.

Bonfanti L., Olive S., Poulain D.A., Theodosis D.T. Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. *Neuroscience*, 1992, 49, 419-436.

Bonfanti L., Theodosis D.T. Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb. *Neuroscience*, 1994, 62, 291-305.

Bonfanti L., Peretto P., Merighi A., Fasolo A. Newly-generated cells from the rostral migratory stream in the accessory olfactory bulb of the adult rat. *Neuroscience*, 1997, 81, 489-502.

Bonfanti L. PSA-NCAM in mammalian structural plasticity and neurogenesis. *Prog. Neurobiol*., 2006, 80, 129-164.

Bonfanti L., Ponti G. Adult mammalian neurogenesis and the New Zealand white rabbit. *Vet. J.*, 2008, 175, 310-331.

Bonfanti L. From hydra regeneration to human brain structural plasticity: a long trip through narrowing roads. *The ScientificWorld Journal*, 2011, 11, 1270–1299.

Bonfanti L., Peretto P. Adult neurogenesis in mammals – a theme with many variations. *Eur. J. Neurosci*., 2011, 34, 930-950.

Bonfanti L., Peretto P. The missing chain. *Front. Neurosci*., 2012, 6, 5.

Bonfanti L. The (real) neurogenic/gliogenic potential of the postnatal and adult brain parenchyma. *ISRN Neuroscience*, 2013, Article ID 354136.

Butt A.M., Hamilton N., Hubbard P., Pugh M., Ibrahim M. Synantocytes: the fifth element. *J. Anat.*, 2005, 207, 695-706.

Cameron H.A., McKay R.D. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J. *Comp. Neurol*., 2001, 435, 406-417.

Carleton A., Petreanu L., Lansford R., Alvarez-Buylla A., Lledo P.M. Becoming a new neuron in the adult olfactory bulb. *Nat. Neurosci*., 2003, 6, 507-518.

Caroni P. Intrinsic neuronal determinants that promote axonal sprouting and elongation. *Bioessays*, 1997, 19, 767-775.

Cheng M.F. Hypothalamic neurogenesis in the adult brain. *Front. Neuroendocrinol.*, 2013, 34, 167-178.

Chklovskii D.B., Mel B.W., Svoboda K. Cortical rewiring and information storage. *Nature*, 2004, 431, 782-788.

Crociara P., Parolisi R., Conte D., Fumagalli M., Bonfanti L. Cellular and molecular characterization of multipolar Map5-expressing cells: A subset of newly generated, stage-specific parenchymal cells in the mammalian central nervous system. *PLoS ONE*, 2013, 8, e63258.

Curtis M.A., Kam M., Nannmark U., Anderson M.F., Axell M.Z., Wikkelso C., Holtas S., van Roon-Mom W.M., Bjork-Eriksson T., Nordborg C., Frisen J., Dragunow M., Faull R.L., Eriksson P.S. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science*, 2007, 315, 1243-1249.

Davies S.J., Fitch M.T., Memberg S.P., Hall A.K., Raisman G., Silver J. Regeneration of adult axons in white matter tracts of the central nervous system. *Nature*, 1997, 390, 680-683.

Dayer A., Cleaver K., Abouantoun T., Cameron H. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. *J. Cell Biol.*, 2005, 168, 415-427.

Dawson M.R., Polito A., Levine J.M., Reynolds R. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. *Mol. Cell. Neurosci.*, 2003, 24, 476-488.

De Marchis S., Temoney S., Erdelyi F., Bovetti S., Bovolin P., Szabo G., Puche A.C. GABAergic phenotypic differentiation of a subpopulation of subventricular derived migrating progenitors. *Eur. J. Neurosci*., 2004, 20, 1307-1317.

Doetsch F., Alvarez-Buylla A. Network of tangential pathways for neuronal migration in adult mammalian brain. *Proc. Natl. Acad. Sci. U.S.A.*, 1996, 93, 14895-14900.

Doetsch F., Garcìa-Verdugo J.M., Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci*., 1997, 17, 5046-5041.

Doetsch F., Caille I., Lim D.A., Garcìa-Verdugo J.M., Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*, 1999, 97, 703-716.

Doetsch F., Hen R. Young and excitable: the function of new neurons in the adult mammalian brain. Curr. Opin. Neurobiol., 2005, 15, 121-128.

Dusart I., Morel M.P., Wehrle R., Sotelo C. Late axonal sprouting of injured Purkinje cells and its temporal correlation with permissive changes in the glial scar. *J. Comp. Neurol*., 1999, 408, 399-418.

Eriksson P.S., Perfilieva E., Biork-Eriksson T., Alborn A.M., Nordborg C., Peterson D.A., Gage F.H. Neurogenesis in the adult human hippocampus. *Nat. Med*., 1998, 4, 1313-1317.

Ferguson M.W.J., O'Kane S. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos. Trans. R. Soc. Lond. B Biol. Sci.,* 2004, 359, 839-850.

Frielingsdorf H., Schwarz K., Brundin P., Mohapel P. No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. *Proc. Natl. Acad. Sci. USA.,* 2004, 101, 10177-10182.

Frotscher M., Specificity of interneuronal connections. *Ann. Anat*., 1992, 174, 377-382.

Gage F.H. Mammalian neural stem cells. *Science*, 2000, 287, 1433-1438.

Garwicz M., Christensson M., Psounib E. A unifying model for timing of walking onset in humans and other mammals. *Proc. Natl. Acad. Sci. U.S.A.*, 2009, 106, 21889-21893.

Gould E., Tanapat P., Hastings N.B., Shors T.J. Neurogenesis in adulthood: a possible role in learning. *Trends Cogn. Sci*., 1999a, 3, 186-192.

Gould E., Reeves A.J., Graziano M.S., Gross C.G. Neurogenesis in the neocortex of adult primates. *Science*, 1999b, 286, 548-552.

Gould E., Vail N., Wagers M., Gross C.G. Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. *Proc. Natl. Acad. Sci. U.S.A.*, 2001, 98, 10910-10917.

Grimaldi P., Parras C., Guillemot F., Rossi F., Wassef M. Origins and control of the differentiation of inhibitory interneurons and glia in the cerebellum. *Dev. Biol*., 2009, 328, 422-433.

Gross C.G. Neurogenesis in the adult brain: death of a dogma. *Nat. Rev. Neurosci*., 2000, 1, 67-73.

Gumbiner B.M. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell, 1996, 84, 345-357.

Guo F., Maeda Y., Ma J., Xu J., Horiuchi M., Miers L., Vaccarino F., Pleasure D. Pyramidal neurons are generated from oligodendroglial progenitor cells in adult piriform cortex. *J. Neurosci.,* 2010, 30, 12036-12049.

Hastings N.B., Gould E. Rapid extension of axons into the CA3 region by adult generated granule cells. *J. Comp. Neurol*., 1999, 413, 146-154.

Holtmaat A., Svoboda K. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat. Rev. Neurosci.,* 2009, 10, 647-658.

Horner P.J., Power A.E., Kempermann G., Kuhn H.G., Palmer T.D., Winkler J., Thal L.J., Gage F.H. Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. *J. Neurosci*., 2000, 20, 2218-2228.

Hu H., Tomasiewicz H., Magnuson T., Rutishauser U. The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone. *Neuron*, 1996, 16, 735-743.

Ihrie R.A., Alvarez-Buylla A. Lake-front property: A unique germinal niche by the lateral ventricles of the adult brain. *Neuron*, 2011, 70, 674-686.

Imayoshi I., Sakamoto M., Ohtsuka T., Takao K., Miyakawa T., Yamaguchi M., Mori K., Ikeda T., Itohara S., Kageyama R. Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat. Neurosci*., 2008, 11, 1153-1161.

Johnson K.M., Boonstra R., Wojtowicz J.M. Hippocampal neurogenesis in food-storing red squirrels: the impact of age and spatial behaviour. *Genes Brain Behav.*, 2010, 9, 583-591.

Kempermann G., Kuhn H.G., Gage F.H. More hippocampal neurons in adult mice living in an enriched environment. *Nature*, 1997, 386, 493-495.

Kempermann G. Why new neurons? Possible functions for adult hippocampal neurogenesis. *J. Neurosci*., 2002, 22, 635-638.

Kempermann G., Gaat D., Kronenberg G., Yamaguchi M., Gage F.H. Early determination and long-tem persistence of adult-generated new neurons in the hippocampus of mice. *Development*, 2003, 130, 391-400.

Kempermann G, Jessberger S, Steiner B, Kronenberg G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci*., 2004, 27, 447-452.

Kempermann G. New neurons for 'survival of the fittest'. *Nat. Rev. Neurosci.*, 2012, 13, 727-736.

Kokaia Z., Lindvall O. Neurogenesis after ischaemic brain insults. *Curr. Op. Neurobiol*., 2003, 13, 127-132.

Kokoeva M.V., Yin H., Flier J.S. Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science*, 2005, 310, 679-683.

Komitova M., Perfilieva E., Mattsson B., Eriksson P.S., Johansson B.B. Enriched environment after focal cortical ischemia enhances the generation of astroglia and NG2 positive polydendrocytes in adult rat neocortex. *Exp. Neurol.*, 2006, 199, 113-121.

Komuro H., Yacubova E., Yacubova E., Rakic P. Mode and tempo of tangential cell migration in the cerebellar external granule layer. *J. Neurosci*., 2001, 21, 527-540.

Konefal S., Elliot M., Crespi B. The adaptive significance of adult neurogenesis: an integrative approach. *Front. Neuroanat*., 2013, 7, 21.

Kornack D.R. Neurogenesis and the evolution of cortical diversity: mode, tempo and partitioning during development and persistence in adulthood. *Brain Behav. Evol*., 2000, 55, 336-344.

Kornack D.R., Rakic P. The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc. Natl. Acad. Sci. U.S.A.*, 2001a, 98, 4752-4757.

Kornack D.R., Rakic P. Cell proliferation without neurogenesis in adult primate neocortex. *Science*, 2001b, 294, 2127-2130.

Kriegstein A., Alvarez-Buylla A. The glial nature of embryonic and adult neural stem cells. *Ann. Rev. Neurosci.*, 2009, 32, 149-184.

Kuhn H.G., Dickinson-Anson H., Gage F.H. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitors proliferation. *J. Neurosci*., 1996, 16, 2027-2033.

Kuhn G.H., Blomgren K. Developmental dysregulation of adult neurogenesis. *Eur. J. Neurosci.*, 2011, 33, 1115-1122.

Kung J.W., Forbes S.J. Stem cells and liver repair. *Curr. Opin. Biotechnol.*, 2009, 20, 568-574.

Leonhardt H. Topographic distribution of subependymal basement labyrinths of the ventricular system of brain in rabbit. *Zeits. Zellfor. Mikr. Anat*., 1972, 127, 392-406.

Leto K., Bartolini A., Yanagawa Y., Obata K., Magrassi L., Schilling K. Rossi F. Laminar fate and phenotype specification of cerebellar GABAergic interneurons. *J. Neurosci*., 2009, 29, 7079-7091.

Leuner B., Gould E., Shors T.J. Is there a link between adult neurogenesis and learning? *Hippocampus*, 2006, 16, 216-224.

Levison S.W. (Ed.) Mammalian subventricular zones. Springer, Newark, USA, 2006.

Lindvall O., Kokaia Z. Stem cells in human neurodegenerative disorders--time for clinical translation? *J. Clin. Invest.*, 2010, 120, 29-40.

Little M.H., Bertram J.F. Is there such a thing as a renal stem cell? *J. Am. Soc. Nephrol.*, 2009, 20, 2112-2117.

Lledo P.M., Alonso M., Grubb M.S. Adult neurogenesis and functional plasticity in neuronal circuits. *Nat. Rev. Neurosci*., 2006, 7, 179-193.

Lois C., Alvarez-Buylla A. Long-distance neuronal migration in the adult mammalian brain. *Science*, 1994, 264, 1145-1148.

Lois C., Garcìa-Verdugo J., Alvarez-Buylla A. Chain migration of neuronal precursors. *Science*, 1996, 271, 978-981.

Luskin M.B. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron*, 1993, 11, 173-189.

Luzzati F., Peretto P., Aimar P., Ponti G., Fasolo A., Bonfanti L. Glia independent chains of neuroblasts through the subcortical parenchyma of the adult rabbit brain. *Proc. Natl. Acad. Sci. U.S.A.*, 2003, 100, 13036-13041.

Luzzati F., De Marchis S., Fasolo A., Peretto P. Neurogenesis in the caudate nucleus of the adult rabbit. *J. Neurosci.*, 2006, 26, 609-621.

Luzzati F., De Marchis S., Parlato R., Gribaudo S., Schütz G., Fasolo A., Peretto P. New striatal neurons in a mouse model of progressive striatal degeneration are generated in both the subventricular zone and the striatal parenchyma. *PLoS One*, 2011, 6, e25088.

Magavi S.S., Leavitt B.R., Macklis J.D. Induction of neurogenesis in the neocertex of adult mice. *Nature*, 2000, 405, 951-955.

Manning P.J., Ringer D.H., Newcomer C.E. (Eds.) The biology of the laboratory rabbit. Academic Press, San Diego, USA, 1994.

Maricich S.M., Herrup K. Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. J. *Neurobiol*., 1999, 41, 281-294.

Maricich S.M., Gilmore E.C., Herrup K. The role of tangential migration in the establishment of mammalian cortex. *Neuron*, 2001, 31, 175-178.

Marin O., Rubenstein J.L. Cell migration in the forebrain. *Ann. Rev. Neurosci*., 2003, 26, 441-483.

Masaki H., Ide H. Regeneration potency of mouse limbs. *Dev. Growth Differ.*, 2007, 49, 89-98.

McFarland W.L., Morgane P.J. Jacobs M.S. Ventricular system of the brain of the dolphin, Tursiops truncatus, with comparative anatomical observations and relations to brain specializations. *J. Comp. Neurol*., 1969, 135, 275-368.

Merkle F.T., Tramontin A.D., Garcìa-Verdugo J.M., Alvarez-Buylla A. Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl. Acad. Sci. U.S.A.*, 2004, 101, 17528-17532.

Mirescu C., Gould E. Stress and adult neurogenesis. *Hippocampus*, 2006, 16, 233-238.

Misson J.P., Austin C.P., Takahashi T., Cepko C.L., Caviness V.S. The alignment of migrating neural cells in relation to the murine neopallial radial glial fiber system. *Cereb. Cortex*, 1991, 1, 221-229.

Morrison S.J., Spradling A.C. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell*, 2008, 132, 598-611.

Nakatomi H., Kuriu T., Okabe S., Yamamoto S., Hatano O., Kawahara N., Tamura A., Kirino T., Nakafuku M. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell*, 2002, 110, 429-441.

Nishiyama A. Polydendrocytes: NG2 cells with many roles in development and repair of the CNS. *Neuroscientist*, 2007, 13, 62-76.

Nishiyama A., Komitova M., Suzuki R., Zhu X. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nat. Rev. Neurosci*., 2009, 10, 9-22.

Nystul T.G., Spradling, A.C. Breaking out of the mold: diversity within adult stem cells and their niches. *Curr. Opin. Genet. Dev.*, 2006, 16, 463-468.

Ohira K., Furuta T., Hioki H., Nakamura K.C., Kuramoto E., Tanaka Y., Funatsu N., Shimizu K., Oishi T., Hayashi M., Miyakawa T., Kaneko T., Nakamura S. Ischemia-induced neurogenesis of neocortical layer 1 progenitor cells. *Nat. Neurosci.*, 2009, 13, 173-179.

Palmer T.D., Markakis E.A., Willhoite A.R., Safar F., Gage F.H. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.*, 1999, 19, 8487-8497.

Parent J.M., Yu T.W., Leibowitz R.T., Geshcwind D.H., Sloviter R.S., Lowenstein D.H. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J. Neurosci*., 1997, 17, 3727-3738.

Peretto P., Merighi A., Fasolo A., Bonfanti L. Glial tubes in the rostral migratory stream of the adult rat. *Brain Res. Bull*., 1997, 42, 9-21.

Peretto P., Giachino C., Aimar P., Fasolo A., Bonfanti L. Chain formation and glial tube assembly in the shift from neonatal to adult subventricular zone of the rodent forebrain. *J. Comp. Neurol*., 2005, 487, 407-427.

Petreanu L., Alvarez-Buylla A. Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. *J. Neurosci*., 2002, 22, 6106-6113.

Pierce A.A., Xu A.W. De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. *J. Neurosci.*, 2010, 30, 723-730.

Ponti G., Aimar P., Bonfanti L. Cellular composition and cytoarchitecture of the rabbit subventricular zone (SVZ) and its extensions in the forebrain. *J. Comp. Neurol.*, 2006a, 498, 491-507.

Ponti G., Peretto P., Bonfanti L. A subpial, transitory germinal zone forms chains of neuronal precursors in the rabbit cerebellum. *Dev. Biol.*, 2006b, 294, 168-180.

Ponti G., Peretto P., Bonfanti L. Genesis of neuronal and glial progenitors in the cerebellar cortex of peripuberal and adult rabbits. *PLoS ONE*, 2008, 3, e2366.

Ponti G., Crociara P., Armentano M, Bonfanti L. Adult neurogenesis without germinal layers: the "atypical" cerebellum of rabbits. *Archiv. Ital. Biol.*, 2010, 148, 147-158.

Ponti G., Obernier K., Guinto C., Jose L., Bonfanti L., Alvarez-Buylla A. Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. *Proc. Natl. Acad. Sci. U.S.A.*, 2013, 110, 1045-1054.

Quinones-Hinojosa A., Sanai N., Soriano-Navarro M., Gonzalez-Perez O., Mirzadeh Z., Gil-Perotin S., Romero-Rodriguez R., Berger M.S., Garcìa-Verdugo J.M., Alvarez-Buylla A.

Cellular composition and cytoarchitecture of the adult human subventricular zone: a niche of neural stem cells. *J. Comp. Neurol*., 2006, 494, 415-434.

Rakic, P. Principles of neural cell migration. *Experientia*, 1990, 46, 882-891.

Rakic P., Ang E.S.B.C., Breunig J. Setting the stage for cognition: Genesis of the primate cerebral cortex. In: Gazzaniga, M.S. (Ed.), The Cognitive Neurosciences III. The MIT Press, Cambridge, USA, 2004, 33-49.

Reynolds B.A., Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, 1992, 255, 1707-1710.

Richardson W.D., Young K.M., Tripathi R.B., McKenzie I. NG2-glia as multipotent neural stem cells: fact or fantasy? *Neuron*, 2011, 70, 661-673.

Riederer B., Cohen R., Matus A. MAP5: a novel brain microtubule associated protein under strong developmental regulation. *J. Neurocytol*., 1986, 15, 763-775.

Riederer B.M. Microtubule-associated protein 1B, a growth and phosphorilated scaffold protein. *Brain Res. Bull*., 2007, 71, 541-558.

Rivers L.E., Young K.M., Rizzi M., Jamen F., Psachoulia K., Wade A., Kessaris N., Richardson W.D. PDGFRA/NG2 glia generatemyelinating oligodendrocytes and piriformprojection neurons in adult mice. *Nat. Neurosci.*, 2008, 11, 1392-1401.

Rodriguez-Perez L.M., Perez-Martin M., Jimenez A.J., Fernandez-Llebrez P. Immunocytochemical characterization of the wall of the bovine ventricle. Cell Tiss. Res., 2003, 314, 325-335.

Sanai N., Tramontin A.D., Quinones-Hinojosa A., Barbaro N.M., Gupta N., Kunwar S., Lawton M.T., McDermott M.W., Parsa A.T., Garcìa Verdugo J.M., Berger M.S., Alvarez-Buylla A. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature, 2004, 427, 740-744.

Sanai N., Nguyen T., Ihrie R. A., Mirzadeh Z., Tsai H-H., Wong M., Gupta N., Berger M-S., Huang E., Garcia-Verdugo J-M., Rowitch D.H., Alvarez-Buylla A. Corridors of migrating neurons in the human brain and their decline during infancy. *Nature*, 2011, 478, 382-386.

Sanchez-Villagra M.R., Sultan F. The cerebellum at birth in therian mammals, with special reference to rodents. Brain Behav. Evol., 2001, 59, 101-113.

Schoenfeld T.A., McKerracher L., Obar R., Vallee R.B. MAP 1A and MAP 1B are structurally related microtubule associated proteins with distinct developmental patterns in the CNS. *J. Neurosci.*, 1989, 9, 1712-1730.

Seri B., Garcìa-Verdugo J.M., Collaudo-Morente L., McEwen B.S., Alvarez-Buylla A. Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *J. Comp. Neurol*., 2004, 478, 359-378.

Simon C., Götz M., Dimou L. Progenitors in the adult cerebral cortex: cell cycle properties and regulation by physiological stimuli and injury. Glia, 2011, 59, 869-881.

Smith K.R. Jr. The cerebellar cortex of the rabbit. An electron microscopic study. *J. Comp. Neurol*., 1963, 121, 459-483.

Sohur U.S., Emsley J.G., Mitchell B.D., Macklis J.D. Adult neurogenesis and cellular brain repair with neural progenitors, precursors and stem cells. *Phil. Trans. Royal Soc. B*, 2006, 361, 1477-1497.

Spacek J., Parizek J., Lieberman A.R. Golgi cells, granule cells and synaptic glomeruli in the molecular layer of the rabbit cerebellar cortex. *J. Neurocytol*., 1973, 2, 407-428.

Spalding K.L., Bergmann O., Alkass K., Bernard S., Salehpour M., Huttner H.B., Boström E., Westerlund I., Vial C., Buchholz B.A., Possnert G., Mash D.C., Druid H., Frisén J. Dynamics of hippocampal neurogenesis in adult humans. *Cell*, 2013, 153, 1219-1227.

Stallcup W.B. The NG2 antigen, a putative lineage marker: immunofluorescent localization in primary cultures of rat brain. *Dev. Biol.*, 1981, 83, 154-165.

Tanapat P., Hastings N.B., Reeves A.J., Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J. Neurosci*., 1999, 19, 5792-5801.

Theodosis D.T., Poulain D.A., Oliet S.H.R. Activity-dependent structural and functional plasticity

of astrocyte-neuron interactions. *Physiol. Rev*., 2008, 88, 983-1008.

Thored P., Arvidsson A., Cacci E., Ahlenius H., Kallur T., Darsalia V., Ekdahl C.T., Kokaia Z., Lindvall O. Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells*, 2006, 24, 739-747.

Tramontin A.D., Garcia-Verdugo J.M., Lim D.A., Alvarez-Buylla A. Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb Cortex*, 2003, 13, 580-587.

Trotter J., Karram K., Nishiyama A. NG2 cells: properties, progeny and origin. *Brain Res. Rev.*, 2010, 63, 72-82.

Van Praag H., Kempermann G., Gage F.H. Running increases cell proliferation and neurogenesis, in the adult mouse dentate gyrus. *Nat. Neurosci*., 1999, 2, 266-270.

Van Praag H., Schinder A.F., Christie B.R., Toni N., Palmer T.D., Gage F.H. Functional neurogenesis in the adult hippocampus. *Nature*, 2002, 415, 1030-1034.

Weisheit G., Gliem M., Endl E., Pfeffer P.L., Busslinger M., Schilling K. Postnatal development of the murine cerebellar cortex: formation and early dispersal of basket, stellate and Golgi neurons. *Eur. J. Neurosci.*, 2006, 24, 466-478.

Witter P.M., Amaral D.G. Hippocampal formation. In: Paxinos, G., (Ed.), The rat nervous system, 3rd Edition. 2004, Academic Press, San Diego, USA, pp. 635-704.

Wright D.E., Wagers A.J., Gulati A.P., Johnson F.L., Weissman I.L. Physiological migration of hematopoietic stem and progenitor cells. *Science*, 2001, 294, 1933-1936.

Yamashita T., Ninomiya M., Hernandez Acosta P., Garcia-Verdugo J.M., Sunabori T., Sakaguchi M., Adachi K., Kojima T., Hirota Y., Kawase T., Araki N., Abe K., Okano H., Sawamoto K. Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *J. Neurosci*., 2006, 26, 6627-6636.

Zhao M., Momma S., Delfani K., Carlen M., Cassidy R.M., Johansson C.B., Brismar H., Shupliakov O., Frisen J., Janson A.M. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc. Natl. Acad. Sci. U.S.A.*, 2003, 100, 7925-7930.

Zhao C., Teng E.M., Summers R.G. Jr., Ming G.L., Gage F.H. Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J. Neurosci*., 2006, 26, 3-11.

Zilles K. Neuronal plasticity as an adaptive property of the central nervous system. *Ann. Anat.*, 1992, 174, 383-391.

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter II

A Survey on the Studies of Rabbit Prion Proteins

Jiapu Zhang*
School of Science, Informatics Technology, and Engineering,
and Centre of Informatics and Applied Optimisation,
The University of Ballarat, MT Helen Campus, Victoria, Australia

Abstract

In 2012 Nov-Dec, an article named "Naturally prion resistant mammals: a utopia?" (Prion 6(5):425-429; Proc Natl Acad Sci USA 109(13):5080-5085, written by Chianini F and Fernández-Borges N et al.) seems to have killed all the articles of the past few decades reporting rabbits are resistant to prion infection. As we all well know, "Multiple amino acid residues within the rabbit prion protein inhibit formation of its abnormal isoform" (J Virol 77(3):2003-2009). Which is right and which is wrong? Thus, at this moment, it is very worth doing a detailed survey on the studies of rabbit prion proteins by this article.

Keywords: Rabbits; Prion Diseases; Immunity; Infected

Introduction

In 2012, "mad rabbit disease" was reported being generated through saPMCA (serial automated Protein Misfolding Cyclic Amplification) in vitro and the rabbit prion generated is infectious and transmissible [1-3]. However, classical studies have showed that rabbits have low susceptibility to be infected by diseased prions (PrP^{Sc}) [4-15] as horses and dogs. Thus, at

*Corresponding author: Phone: 61-423487360, 61-3-5327 6335, Email: jiapu_zhang@hotmail.com, j.zhang@ballarat.edu.au.

this moment, this article carries an important task to do a detailed survey on the studies of rabbit prion protein (RaPrP).

The Detailed Survey

In this section, we do the survey accroding to research advancements of RaPrP research from the Year 1976. Scrapie is a prion disease in sheep and goats. In 1976, Barlow and Rennie (1976) made many attempts to infect rabbits with the ME7 scrapie and other known prion strains but all failed at last [6].

In 1984~1985, some antibodies to the scrapie protein were reported by Prusiner's research group [16,17]. The antibodies to the scrapie agent were produced after immunization of rabbits with either scrapie prions or the prion protein PrP(27-30). The monospecificity of the rabbit antiserum raised against PrP(27-30) was established by its reactivity after affinity purification, and the rabbit antiserum to PrP(27-30) was successfully produced. In 1985, the characterization of antisera raised in rabbits, against scrapie-associated prion diseases and the prion human Creutzfeldt-Jakob disease (CJD), was studied in [18].

In 1986, Takahashi et al. (1986) reported, rabbits immunized with the fraction P4 containing scrapie infectivity prepared from mouse brains raised antibodies against three major polypeptides of [19]. Cho (1986) reported that the antibody to scrapie-associated fibril protein roduced in a rabbit identifies a cellular antigen [20]. Barry et al. (1986) reported that rabbit antisera to a synthetic peptide PrP-P1 constructed based on PrP(27-30) were found by immunoblotting to react with PrP(27-30) and its precursor PrP^{Sc}(33-35), as well as with a related protease-sensitive cellular homologue PrP^{C}(33-35), this means scrapie (PrP^{Sc}) and cellular (PrP^{C}) prion proteins share polypeptide epitopes [21]. An enzyme-linked immunosorbent assay showed that rabbit antiserum to PrP(27-30) was more reactive with PrP(27-30) than with PrP-P1; conversely, antiserum to PrP-P1 was more reactive with the peptide than with the prion proteins [21]. Shinagawa et al. (1986) reported "immunization of a rabbit with the (synthetic) peptide conjugated with ovalbumin induced specific antibodies" corresponding to the N-terminal region of the scrapie prion protein (PrP^{Sc}) [22]. "Rabbit antisera were raised to SAFs (scrapie-associated fibrils) isolated from mice infected with the ME7 scrapie strain and to SAFs isolated from hamsters infected with the 263K scrapie strain" [23,24]. Robakis et al. (1986) clearly pointed out that rabbit brain is resistant to scrapie infection [25] in 1986.

In 1987, there were several reports on rabbits. Bockman et al. (1987) identified by immunoblotting human & mouse CJD prion proteins ($HuPrP^{Sc}$ and $MoPrP^{Sc}$) using rabbit antisera raised against hamster scrapie prion proteins ($HaPrP^{Sc}$) [26]. Wade et al. (1987) found a 45 kD protein in scrapie-infected hamster brain has a signal to inoculate rabbits [27]. Kascsak et al. (1987) reported "MAb (monoclonal antibody) 263K 3F4 (that was derived from a mouse immunized with hamster 263K PrP^{Sc} reacted with hamster but not mouse PrP^{Sc}) recognized normal host protein of 33 to 35 kilodaltons in brain tissue from hamsters and humans but not from bovine, mouse, rat, sheep, or rabbit brains" [28]. Wiley et al. (1987) used rabbit monospecific antisera raised against synthetic peptides corresponding to the N-terminal 13 or 15 amino acids of PrP(27-30) and rabbit antisera raised against infectious prions or PrP(27-30) purified from scrapie-infected hamster brains to immunostaine

glutaraldehyde-perfused hamster brains [29]. Hay et al. (1987) found the evidence for a secretory form of the cellular prion protein (PrP^C) "cell-free translation studies in rabbit reticulocyte lysates supplemented with microsomal membranes gave results: while one form of HaPrP (hamster brain prion protein) was found as an integral membrane protein spanning the membrane at least twice, another form of HaPrP was found to be completely translocated to the microsomal membrane vesicle lumen" [30].

In 1998, Caughey et al. (1988) detected the immunoprecipitation of PrP synthesis using a rabbit antibody specific for a 15 amino acid PrP peptide and concluded that "either PrP is not the transmissible agent of scrapie or the PrP is not processed appropriately in this cell system to create the infectious agent" [31]. Barry et al. (1988) undertook ELISA (enzyme-linked immunosorbent assay) and immunoblotting studies with rabbit antisera raised against three synthetic PrP peptides of PrP(27-30), PrP^{Sc}, and PrP^C and concluded that the three proteins are encoded by the same chromosomal gene [32]. Baron et al. (1998) found "polyclonal rabbit antiserum to SAF protein was reacted with brain sections from scrapie-infected mice, two familial cases of transmissible dementia, and three cases of Alzheimer's disease (AD)" and "evidence of the similarity of SAF protein to PrP(27-30)" [33]. Gabizon et al. (1988) found "polyclonal RaPrP antiserum raised against NaDodSO4/PAGE-purified scrapie prion protein of 27-30 kDa reduced scrapie infectivity dispersed into detergent-lipid-protein complexes" [34]. Roberts et al. (1998), "using monoclonal antibodies to a synthetic peptide corresponding to a portion of beta-protein and rabbit antiserum to hamster scrapie PrP(27-30), examined in situ amyloid plaques on sections from cases of neurodegenerative diseases" and their "results emphasize the need for classification of CNS (central nervous system) amyloids based on the macromolecular components comprising these pathologic polymers" [35].

In 1989, Gabizon et al. (1989) reported that "polyclonal rabbit PrP antiserum raised against sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-purified PrP(27-30) reduced scrapie infectivity dispersed into DLPC (detergent-lipid-protein complexes)" [36]. "Kuru plaque is a pathognomonic feature in the brain of patients with CJD and in the brain of CJD-infected mice" [37], Kitamoto et al. (1989) reported "kuru plaques from CJD-infected mice were immunolabeled with rabbit anti-murine prion protein (PrP) absorbed with human PrP, but not so with mouse anti-human PrP" [37]. Farquhar et al. (1989) "Two polyclonal antisera were raised in rabbits against the scrapie-associated fibril protein (PrP) prepared from sheep and mice" [38].

In 1990, Yost et al. (1990) reported that in the rabbit reticulocyte lysate system, an unusual topogenic sequence in the prion protein fails to cause stop transfer (the polypeptide chain across the membrane of the endoplasmic reticulum) of most nascent chains [39]. Lopez et al. (1990) reported a completely translocated (secretory) topology form of the major product synthesized in rabbit reticulocyte lysates (RRL) [40].

In 1991, Di Martino et al. (1991) reported the characterization of two polyclonal antibodies which were raised by immunizing rabbits with two non carrier-linked synthetic peptides whose amino acid sequences corresponded to codons 89-107 (peptide P1) and 219-233 (peptide P2) of the translated cDNA sequence of murine PrP protein [41]. Ikegami et al. (1991) detected the scrapie-associated fibrillar protein in the lymphoreticular organs of sheep by means of a rabbit-anti-sheep PrP (the scrapie-associated fibrillar protein) polyclonal antibody by Western blot analysis [42].

In 1992, Hashimoto et al. (1992) did immunohistochemical study of kuru plaques using antibodies against synthetic prion protein peptides. Two synthetic peptides were used to

immunize rabbits and produce antisera (anti-N and anti-M) [43]. Kirkwood et al. (1992) using rabbit antiserum raised against mouse PrP detected an abnormal PrP (prion protein) from the brains of domestic cattle with spongiform encephalopathy (SE) [44].

In 1993, the Western blot analysis was performed with rabbit serum against the sheep SAF [45]. To determine if amyloid deposits be visualized by immunocytochemical techniques, Guiroy et al. (1993) used a rabbit antiserum directed against scrapie amyloid (PrP27-30) to stain formalin-fixed, formic acid-treated brain tissue sections from several animal species with natural and experimental transmissible mink encephalopathy (TME) [46]. Groschup and Pfaff (1993) reported that "rabbit antisera to synthetic peptides representing amino acid sequence 108 to 123 of PrP of cattle, sheep and mice reacted strongly with modified PrP of the homologous host but not, or only poorly, with PrP of heterogeneous origin" [47]. Miller et al. (1993) did the immunohistochemical detection of prion protein in sheep with scrapie using a primary antibody obtained from a rabbit immunized to PrP^{Sc} extracted from brains of mice with experimentally induced scrapie [48].

In 1994, Groschup et al. (1994) investigated with eight different anti-peptide sera raised in rabbits against various synthetic peptides representing segments of the amino acid (aa) sequence 101-122 of ovine, bovine, murine and hamster PrP, and found that "the region close to the actual or putative proteinase K cleavage sites of PrP seems to exhibit high structural variability among mammalian species" [49]. Xi et al. (1994) detected the proteinase-resistant protein (PrP) in small brain tissue samples from CJD patients using rabbit polyclonal antibody against hamster PrP(27-30) [50]. Schmerr et al. (1994) used a fluorescein-labeled goat anti-rabbit immunoglobulin as an antibody and used rabbit antiserum for immunoblot analysis, and PrP^{Sc} was solubilized and reacted with a rabbit antiserum specific for a peptide of the prion protein [51].

In 1995, Yokoyama et al. (1995) used antisera raised in rabbits against three peptides PrP 150-159, PrP 165-174, and PrP 213-226 of mouse prion and concluded that rabbit antiserum against the MAP (multiple antigenic peptide) representing amino acid sequence 213-226 of mouse PrP is useful as a diagnostic tool for prion disease of animals [52].

In 1996, Yokoyama et al. (1996) detected species specific epitopes of mouse and hamster prion proteins by anti-peptide antibodies, where the antisera were produced in rabbits [53].

In 1997, Madec et al. (1997) undertook Western blot analyses using rabbit antiserum that recognized both normal and pathologic sheep prion proteins to study the biochemical properties of PrP^{Sc} in natural sheep scrapie [54]. Loftus and Rogers (1997) cloned RaPrP open reading frame (ORF) and characterised rabbits as a species with apparent resistance to infection by prions [55]. Groschup et al. (1997) raised antisera in rabbits and chicken against sixteen synthetic peptides which represent the complete amino acid sequence of ovine PrP to generate antibodies to further regions of PrP, in order to immunochemical diagnosis and pathogenetic studies on prion diseases [56]. In [57], "the Ure2p yeast prion-like protein was translated in vitro in the presence of labeled [35S]methionine in either rabbit reticulocyte lysate (RRL) or wheat germ extract (WGE) cell-free systems". In 1997, Korth et al. (1997) found that RaPrP was not recognized by a conformational antibody specific for PrP^{Sc}-like structures [8].

In 1999, Takahashi et al. (1999) immunized rabbits with four synthetic peptides and compared the immunoreactivity of antibodies to bovine prion proteins (bovine-PrPs) from various species by immunoblotting and immunohistochemistry [58] and they identified two regions in bovine-PrP which appear suitable for raising antibodies that detect various kinds of

PrPs, and one region (Ab103-121) which appears suitable for raising antibodies that detect several species of PrPs [58].

In 2000, Garssen et al. (2000) did applicability of three anti-PrP peptide sera including staining of tonsils and brainstem of sheep with scrapie [59]. "The three rabbit antibodies (R521, R505, R524) were produced, and raised to synthetic peptides corresponding to residues 94-105, 100-111, and 223-234, respectively, of the sheep prion protein" [59]. "The usefulness of all three anti-peptide sera in the immunohistochemical detection of PrPSc in brain stem and tonsils of scrapie-affected sheep was demonstrated and compared with an established rabbit anti-PrP serum" [59]. Zhao et al. (2000) using prokaryotic expressed GST-PrP fusion protein as antigen, found that "rabbits were immunized subcutaneously" [60].

In 2001, Kelker et al. (2001) showed that "combination of authentic rabbit muscle GAPDH (glyceraldehyde-3-phosphate dehydrogenase) with tNOX (a cell surface NADH oxidase of cancer cells) renders the GAPDH resistant to proteinase K digestion" [61]. Vol'pina et al. (2001) reported "rabbits were immunized with either free peptides or peptide-protein conjugates to result in sera with a high level of antipeptide antibodies" to the bovine spongiform encephalopathy (BSE) prion disease [62]. Bencsik et al. (2001) identified prion protein PrP "using either RB1 rabbit antiserum or 4F2 monoclonal antibody directed against AA 108-123 portion of the bovine and AA 79-92 of human prion protein respectively" and "showed the close vicinity of these PrP expressing cells with noradrenergic fibers" [63]. In [64], "the rabbits were immuned with bovine prion protein ($BoPrP^{C}$) which was expressed in E. coli and anti-PrPC antibody (T1) was obtained", and Li et al. (2001) could detect BSE and scrapie with T1 antibody [64].

In 2002, Laude et al. (2002) reported "In one otherwise refractory rabbit epithelial cell line, a regulable expression of ovine PrP was achieved and found to enable an efficient replication of the scrapie agent in inoculated cultures" [65]. Takekida et al. (2002) established a competitive ELISA to detect prion protein in food products using rabbit polyclonal antibodies that were raised against bovine prion peptides [66].

In 2003, Vorberg et al. (2003) found multiple amino acid residues (such as GLY99, MET108, SER173, ILE214) within the RaPrP inhibit formation of its abnormal isoform [4]. The authors made some substitutions of mouse PrP amino acid sequence by rabbit PrP amino acid sequence and found (i) at the N-terminal region (residues 1-111) the PrP^{Sc} formation is totally prevented, (ii) at the central region (residues 112-177), the constructed PrP failed to be converted to protease resistance, (iii) at the C-terminal region (residues 178-254) the formation of PrP^{Sc} is drastically decreased but is not abolished completely [4]. Thus, rabbit cells are negatively affected by the formation of PrP^{Sc}. Jackman and Schmerr (2003) synthesized fluorescent peptides from the prion protein and produced the corresponding antibodies in rabbits against these peptides, and at last detected abnormal prion protein in a tissue sample [67]. Gilch et al. (2003) reported "treatment of prion-infected mouse cells with polyclonal anti-PrP antibodies generated in rabbit or auto-antibodies produced in mice significantly inhibited endogenous PrP^{Sc} synthesis" and found "immune responses against different epitopes when comparing antibodies induced in rabbits and PrP wild-type mice" [68].

In 2004, Brun et al. (2004) reported the development and further characterisation of a novel PrP-specific monoclonal antibody 2A11, which reacts with PrP^{C} from a variety of species including rabbit [69]. Sachsamanoglou et al. (2004) described "the quality of a rabbit polyclonal antiserum (Sal1) that was raised against mature human recombinant prion protein

(rHuPrP)" [70]. Senator et al. (2004) investigated "the effects of cellular prion protein (PrP^C) overexpression on paraquat-induced toxicity by using an established model system, rabbit kidney epithelial A74 cells, which express a doxycycline-inducible murine PrP^C gene" [71].

In 2005, Golańska et al. (2005) used 2 different anti-14-3-3 antibodies: rabbit polyclonal and mouse monoclonal antibodies to analyze the 14-3-3 protein in the cerebrospinal fluid in CJD [72].

In 2006, Dupiereux et al. (2006) investigated the effect of PrP(106-126) peptide on an established non neuronal model, rabbit kidney epithelial A74 cells that express a doxycycline-inducible murine PrPC gene [73]. Biswas et al. (2006) reported "a rabbit polyclonal anti-serum raised against dimeric MuPrP (murine prion protein) cross-reacted with p46 (a 46 kDa species) and localized the signal within the Golgi apparatus" [74]. Gao et al. (2006) reported recombinant neural protein PrP can bind with both recombinant and native apolipoprotein E (ApoE) in vitro, where the "ApoE-specific antiserum was prepared by immunizing rabbits with the purified ApoE3" [75]. Kocisko and Caughey (2006) reported "rabbit epithelial cells that produce sheep prion protein in the presence of doxycycline (Rov9) have been infected with sheep scrapie" [76]. Xiao et al. (2006) used the method "two male rabbits were immunized for 4 times with the purified protein, and the antiserum against NSE protein was collected and evaluated by ELISA, Western blotting and immunohistochemistry" and concluded "high expression of HuNSE (human neuron-specific enolase) is obtained in E. coli and the prepared antiserum against HuNSE can be used potentially for diagnosis of prion-associated diseases and other nervous degeneration diseases" [77].

In 2007, Oboznaya et al. (2007) reported "antibodies to a nonconjugated prion protein peptide 95-123 interfere with PrP^{Sc} propagation in prion-infected cells", where "rabbits were immunized with free nonconjugated peptides" [78]. Handisurya et al. (2007) reported "Immunization with PrP-virus-like particles induced high-titer antibodies to PrP in rabbit and in rat, without inducing overt adverse effects. As determined by peptide-specific ELISA, rabbit immune sera recognized the inserted murine/rat epitope and also cross-reacted with the homologous rabbit/human epitope differing in one amino acid residue. Rabbit anti-PrP serum contained high-affinity antibody that inhibited de novo synthesis of PrP^{Sc} in prion-infected cells" [79]. Bastian et al. (2007) did an experiment "spiroplasma mirum, a rabbit tick isolate that had previously been shown to experimentally induce spongiform encephalopathy in rodents, was inoculated intracranially (IC) into ruminants" and at last concluded "Spiroplasma spp. from transmissible spongiform encephalopathy (TSE) brains or ticks induce spongiform encephalopathy in ruminants" [80]. Dong et al. (2007) did the interaction analysis between various PrP fusion proteins and the tubulin in vitro, where the native tubulin was extracted from rabbit brian tissues [81].

In 2008, it was said that "ovine prion protein renders rabbit epithelial RK13 cells permissive to the multiplication of ovine prions, thus providing evidence that species barriers can be crossed in cultured cells through the expression of a relevant ovine PrP^C," [82]. Sakudo et al. (2008) "developed a mammalian expression system for a truncated soluble form of human prion protein with the native signal peptide but without a glycosylphosphatidylinositol (GPI)-anchor site, driven by the peptide chain elongation factor 1alpha promoter in stably transfected rabbit-kidney epithelial RK13 cells, to investigate the SOD (superoxide dismutase) activity of mammalian prion protein" and concluded "GPI-anchorless human prion protein is secreted and glycosylated but lacks superoxide dismutase activity" [83]. Shin et al. (2008) cloned a prion protein (PrP) Glu218Lys gene from Korean bovine (Bos taurus

coreanae) and raised the production of rabbit anti-bovine PrP antibody [84]. Lawson et al. (2008) reported "rabbit kidney epithelial cells (RK13) are permissive to infection with prions from a variety of species upon expression of cognate PrP transgenes" [85].

In 2009, Hanoux et al. (2009) reported "when injected into rabbits, (a synthetic peptide) CDR3L generated anti-SAF61 anti-Id polyclonal antibodies that exclusively recognized SAF61 mAb but were unable to compete with hPrP for antibody binding" [86]. Tang et al. (2009) reported fibrinogen, one of the most abundant extracellular proteins, has chaperone-like activity: it maintains thermal-denatured luciferase in a refolding competent state allowing luciferase to be refolded in cooperation with rabbit reticulocyte lysate, and it also inhibits fibril formation of yeast prion protein Sup35 (NM) [87]. Differed from the reaction with N-terminal proline/glycine-rich repeats recognizing rabbit polyclonal antibody, seven monoclonal antibodies (mAbs) against chicken cellular prion protein ($ChPrP^{C}$) were obtained by Ishiguro et al. [88]. Fernandez-Funez et al. (2009) found RaPrP does not induce neurodegeneration in the brains of transgenic flies [7].

In 2010, Nisbet et al. (2010) created a mutant mouse PrP model containing RaPrP specific amino acids at the GPI anchor site and found that the GPI anchor attachment site (ω site) controls the ability of $PrP^{C} \rightarrow PrP^{Sc}$ and the residues at ω and ω+1 of PrP are important modulators of this pathogenic process [11]. Nisbet et al. (2010) recognized that "rabbits are one of a small number of mammalian species reported to be resistant to prion infection" [11]. Wen et al. (2010) using multidimensional heteronuclear NMR techniques reported that the I214V and S173N substitutions result in distinct structural changes for $RaPrP^{C}$ [12, 13] and concluded that the highly ordered β2-α2 loop may contribute to the local as well as global stability of the RaPrP protein [13]. Wen et al. (2010) also recognized "rabbits are one of the few mammalian species that appear to be resistant to TSEs due to the structural characteristics of $RaPrP^{C}$ itself" [13]. Fernandez-Funez et al. (2010) showed that "RaPrP does not induce spongiform degeneration and does not convert into scrapie-like conformers" [89]. Bitel et al. (2010) examined "changes in muscle tissue in a classic model of diabetes and hyperglycemia in rabbits to determine if similar dysregulation of Alzheimer Aβ peptides, the prion protein (PrP), and superoxide dismutase 1 (SOD1), as well as nitric oxide synthases is produced in muscle in diabetic animals" [90]. Khan et al. (2010) found the propensity to form β-state (the β-sheet-rich structure) is greatest for hamster PrP, less for mouse PrP, but least for the PrP of rabbits, horses and dogs under different conditions and using two-wavelength CD (Circular Dichroism) method they also found a key hydrophobic staple-like helix-capping motif keeping the stability of RaPrP's X-ray crystallographic molecular structure [5].

In 2011, Zocche et al. (2011) used the methods "rabbit aortic smooth muscle cells were challenged for 4, 8 and 18 hours, with angiotensin-II, tunicamycin and 7-ketocholesterol, and rabbit aortic arteries were subjected to injury by balloon catheter", and got the results "the PrP^{C} mRNA expression in rabbit aortic artery fragments, subjected to balloon catheter injury, showed a pronounced increase immediately after overdistension" [91]. Mays et al. (2011) reported "PrP^{Sc} was efficiently amplified with lysate of rabbit kidney epithelial RK13 cells stably transfected with the mouse or Syrian hamster PrP gene" [92]. Julien et al. (2011) reported the different overall sensitivities toward NMR urea denaturation with stabilities in the order hamster ≤ mouse < rabbit < bovine protein, and they also investigated the effect of the S174N mutation in rabbit PrP^{C} [93]. Zhou et al. (2011) found that the crowded physiological agents Ficoll 70 and dextran 70 have effects significantly inhibiting fibrillation of rabbit prion protein [14,15]. Fernandez-Funez et al. (2011) also acknowledged that

"Classic studies showing the different susceptibility to prion disease in mammals have recently found support in structural and transgenic studies with PrP from susceptible (mouse, hamster) and resistant (rabbit, horse, dog) animals" [94].

In 2012, Chianini et al. (2012) generated rabbit PrP^{Sc} in vitro subjecting unseeded normal rabbit brain homogenate to saPMCA and found the rabbit PrP^{Sc} generated in vitro is infectious and transmissible [2] and they declared "rabbits are not resistant to prion infection" [2]. Kim et al. (2012) reported "elk prion protein ($ElkPrP^{C}$) has been confirmed to be capable of rendering rabbit epithelial RK13 cells permissive to temporal infection by chronic wasting disease (CWD) prions." [95]. Fernández-Borges et al. (2012) reported the results of [2] and pointed out it is not reasonable to attribute species-specific prion disease resistance based purely on the absence of natural cases and incomplete in vivo challenges; the concept of species resistance to prion disease should be re-evaluated using the new powerful tools available in modern prion laboratories, whether any other species could be at risk [3].

In 2013, Vidal et al. (2013) studied the saPMCA and reported that rabbits are an apparently resistant species to the original classical cattle BSE prion [96]. Wang et al. (2013) reported rabbits are "insensitivity to prion diseases" [97]. Wang et al. (2013) aimed to investigate "potential mechanisms contributing to prion resistance/susceptibility by using the rabbit, a species unsusceptible to prion infection, as a model" [98] and investigated "the expression level and distribution of LRP/LR (laminin receptor precursor/laminin receptor) in rabbit tissues by real-time polymerase chain reaction and by immunochemical analysis with a monoclonal anti-67 kDa LR antibody" [98] and at last their findings confirmed the prion resistance in rabbits [98]. Sweeting et al. (2013) produced X-ray structures of mutants in the β2-α2 loop and reported that the helix-capping motif in the β2-α2 loop modulates β-state misfolding in RaPrP, and still acknowledged "rabbit PrP, a resistant species" [99].

Conclusion

In this article, we focused on the research advances in rabbit prion protein (RaPrP) to give a detailed review. We noticed that the rabbit prion in [2,3] was just produced through saPMCA in vitro not by challenging rabbits directly in vivo with other known prion strains, and the saPMCA result of [2,3] was refused by the test of cattle BSE [96]. All other RaPrP research results generally agree with each other to look rabbits as a resistant species to prion diseases.

References

[1] Urtasun, J. (2012). Mad rabbit disease. *Basqueresearch.com 2012/03/15 News:* http://www.basqueresearch.com/berria_irakurri.asp?Berri_Kod=3859&hizk=I#.Ud397W1yRY4

[2] Chianini, F., Fernndez-Borges, N., Vidal, E., Gibbard, L., Pintado, B., de Castro, J., Priola, S. A., Hamilton, S., Eatona, L. S., Finlayson, J., Pang, Y., Steele, P., Reid, H. W., Dagleish, M. P. & Castilla, J. (2012). Rabbits are not resistant to prion infection. *Proc Natl Acad Sci USA, 109*(13), 5080-5.

[3] Fernández-Borges, N., Chianini, F., Eraña, H., Vidal, E., Eaton, S. L., Pintado, B., Finlayson, J., Dagleish, M. P. & Castilla, J. (2012). Naturally prion resistant mammals: a utopia? *Prion*, *6*(5), 425-9.

[4] Vorberg, I., Martin, H. G., Eberhard, P. & Suzette, A. P. (2003). Multiple amino acid residues within the rabbit prion protein inhibit formation of its abnormal isoform. *J Virol*, *77*(3), 2003-9.

[5] Khan, M. Q., Sweeting, B., Mulligan, V. K., Arslan, P. E., Cashman, N. R., Pai, E. F. & Chakrabartty, A. (2010). Prion disease susceptibility is affected by β-structure folding propensity and local side-chain interactions in PrP. *Proc Natl Acad Sci USA*, *107*(46), 19808-13.

[6] Barlow, R. M. & Rennie, J. C. (1976). The fate of ME7 scrapie infection in rats, guinea-pigs and rabbits. *Res Vet Sci, 21*(1), 110-1.

[7] Fernandez-Funez, P., Casas-Tinto, S., Zhang, Y., Gomez-Velazquez, M., Morales-Garza, M. A., Cepeda-Nieto, A. C., Castilla, J., Soto, C. & Rincon-Limas, D. E. (2009). In vivo generation of neurotoxic prion protein: role for hsp70 in accumulation of misfolded isoforms. *PLoS Genet*, *5*(6), e1000507.

[8] Korth, C., Stierli, B., Streit, P., Moser, M., Schaller, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wthrich, K. & Oesch, B. (1997). Prion (PrP^{Sc})-specific epitope defined by a monoclonal antibody. *Nature*, *390*(6655), 74-7.

[9] Courageot, M. P., Daude, N., Nonno, R., Paquet, S., Di Bari, M. A., Le Dur, A., Chapuis, J., Hill, A. F., Agrimi, U., Laude, H. & Vilette, D. (2008). A cell line infectible by prion strains from different species. *J Gen Virol*, *89*(Pt 1), 341-7.

[10] Vilette, D., Andreoletti, O., Archer, F., Madelaine, M. F., Vilotte, J. L., Lehmann, S. & Laude, H. (2001). Ex vivo propagation of infectious sheep scrapie agent in heterologous epithelial cells expressing ovine prion protein. *Proc Natl Acad Sci USA*, *98*(7), 4055-9.

[11] Nisbet, R. M., Harrison, C. F., Lawson, V. A., Masters, C. L., Cappai, R. & Hill, A. F. (2010). Residues surrounding the glycosylphosphatidylinositol anchor attachment site of PrP modulate prion infection: insight from the resistance of rabbits to prion disease. *J Virol*, *84*(13), 6678-86.

[12] Wen, Y., Li, J., Xiong, M., Peng, Y., Yao, W., Hong, J. & Lin, D. (2010). Solution structure and dynamics of the I214V mutant of the rabbit prion protein. *PLoS One*, *5*(10), e13273.

[13] Wen, Y., Li, J., Yao, W., Xiong, M., Hong, J., Peng, Y., Xiao, G. & Lin, D. (2010). Unique structural characteristics of the rabbit prion protein. *J Biol Chem*, *285*(41), 31682-93.

[14] Zhou, Z., Yan, X., Pan, K., Chen, J., Xie, Z. S., Xiao, G. F., Yang, F. Q. & Liang, Y. (2011). Fibril formation of the rabbit/human/bovine prion proteins. *Biophys J*, *101*(6), 1483-92.

[15] Ma, Q., Fan, J. B., Zhou, Z., Zhou, B. R., Meng, S. R., Hu, J. Y., Chen, J. & Liang, Y., (2012). The contrasting effect of macromolecular crowding on amyloid fibril formation. *PLoS One*, *7*(4), e36288.

[16] Bendheim, P. E., Barry, R. A., DeArmond , S. J., Stites, D. P. & Prusiner, S. B. (1984). Antibodies to a scrapie prion protein. *Nature*, 310(5976), 418-21.

[17] Barry, R. A., McKinley, M. P., Bendheim, P. E., Lewis, G. K., DeArmond, S. J. & Prusiner, S. B., (1985). Antibodies to the scrapie protein decorate prion rods. *J Immunol*, *135*(1), 603-13.

[18] Bode, L., Pocchiari, M, Gelderblom, H. & Diringer, H. (1985). Characterization of antisera against scrapie-associated fibrils (SAF) from affected hamster and cross-reactivity with SAF from scrapie-affected mice and from patients with Creutzfeldt-Jakob disease. *J Gen Virol*, *66*(Pt 11), 2471-8.

[19] Takahashi, K., Shinagawa, M., Doi, S., Sasaki, S., Goto, H. & Sato, G. (1986). Purification of scrapie agent from infected animal brains and raising of antibodies to the purified fraction. *Microbiol Immunol*, *30*(2), 123-31.

[20] Cho, H. J. (1986). Antibody to scrapie-associated fibril protein identifies a cellular antigen. *J Gen Virol*, *67*(Pt 2), 243-53.

[21] Barry, R. A., Kent, S. B., McKinley, M. P., Meyer, R. K., DeArmond, S. J., Hood, L. E. & Prusiner S. B. (1986). Scrapie and cellular prion proteins share polypeptide epitopes. *J Infect Dis*, *153*(5), 848-54.

[22] Shinagawa, M., Munekata, E., Doi, S., Takahashi, K., Goto, H. & Sato, G. (1986). Immunoreactivity of a synthetic pentadecapeptide corresponding to the N-terminal region of the scrapie prion protein. *J Gen Virol*, *67* (Pt 8), 1745-50.

[23] Kascsak, R. J., Rubenstein, R., Merz, P. A., Carp, R. I., Robakis, N. K., Wisniewski, H. M. & Diringer, H. (1986). Immunological comparison of scrapie-associated fibrils isolated from animals infected with four different scrapie strains. *J Virol*, *59*(3), 676-83.

[24] Merz, P. A., Kascsak, R. J., Rubenstein, R., Carp, R. I. & Wisniewski, H. M. (1987). Antisera to scrapie-associated fibril protein and prion protein decorate scrapie-associated fibrils. *J Virol*, *61*(1), 42-9.

[25] Robakis, N. K., Sawh, P. R., Wolfe, G. C., Rubenstein, R., Carp, R. I. & Innis, M. A. (1986). Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *Proc Natl Acad Sci U S A*, *83*(17), 6377-81.

[26] Bockman, J. M., Prusiner, S. B., Tateishi, J. & Kingsbury, D. T. (1987). Immunoblotting of Creutzfeldt-Jakob disease prion proteins: host species-specific epitopes. *Ann Neurol*, *21*(6), 589-95.

[27] Wade, W. F., Dees, C., German, T. L. & Marsh, R. F. (1987). Immunochemical characterization of proteins from scrapie-infected hamster brain, using immunoblot analysis. *Am J Vet Res*, *48*(7), 1077-81.

[28] Kascsak, R. J., Rubenstein, R., Merz, P. A., Tonna-DeMasi, M., Fersko, R., Carp, R. I., Wisniewski, H. M. & Diringer, H. (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J Virol*, *61*(12), 3688-93.

[29] Wiley, C. A., Burrola, P. G., Buchmeier, M. J., Wooddell, M. K., Barry, R. A., Prusiner, S. B., Lampert, P. W. (1987). Immuno-gold localization of prion filaments in scrapie-infected hamster brains. *Lab Invest*, *57*(6), 646-56.

[30] Hay, B., Prusiner, S. B., Lingappa, V. R. (1987). Evidence for a secretory form of the cellular prion protein. *Biochemistry*, *26*(25), 8110-5.

[31] Caughey, B., Race, R., Vogel, M., Buchmeier, M. & Chesebro, B. (1988). In vitro expression of cloned PrP cDNA derived from scrapie-infected mouse brain: lack of transmission of scrapie infectivity. *Ciba Found Symp*, *135*, 197-208.

[32] Barry, R. A., Vincent, M. T., Kent, S. B., Hood, L. E. & Prusiner, S. B. (1988). Characterization of prion proteins with monospecific antisera to synthetic peptides. *J Immunol*, *140*(4), 1188-93.

[33] Baron, H., Baron-Van Evercooren, A. & Brucher, J. M. (1988). Antiserum to scrapie-associated fibril protein reacts with amyloid plaques in familial transmissible dementia. *J Neuropathol Exp Neurol*, *47*(2), 158-65.

[34] Gabizon, R., McKinley, M. P., Groth, D. & Prusiner, S. B. (1988). Immunoaffinity purification and neutralization of scrapie prion infectivity. *Proc Natl Acad Sci U S A*, *85*(18), 6617-21.

[35] Roberts, G. W., Lofthouse, R., Allsop, D., Landon, M., Kidd, M., Prusiner, S. B. & Crow, T. J. (1988). CNS amyloid proteins in neurodegenerative diseases. *Neurology*, *38*(10), 1534-40.

[36] Gabizon, R., McKinley, M. P., Groth, D., Westaway, D., DeArmond, S. J., Carlson, G. A. & Prusiner, S. B. (1989). Immunoaffinity purification and neutralization of scrapie prions. *Prog Clin Biol Res*, *317*, 583-600.

[37] Kitamoto, T., Tateishi, J., Sawa, H. & Doh-Ura, K. (1989). Positive transmission of Creutzfeldt-Jakob disease verified by murine kuru plaques. *Lab Invest*, *60*(4), 507-12.

[38] Farquhar, C. F., Somerville, R. A. & Ritchie, L. A. (1989). Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J Virol Methods*, *24*(1-2), 215-21.

[39] Yost, C. S., Lopez, C. D., Prusiner, S. B., Myers, R. M. & Lingappa, V. R. (1990). Non-hydrophobic extracytoplasmic determinant of stop transfer in the prion protein. *Nature*, *343*(6259), 669-72.

[40] Lopez, C. D., Yost, C. S., Prusiner, S. B., Myers, R. M. & Lingappa, V. R. (1990). Unusual topogenic sequence directs prion protein biogenesis. *Science*, *248*(4952), 226-9.

[41] Di Martino, A., Bigon, E., Corona, G. & Callegaro, L. (1991). Production and characterization of antibodies to mouse scrapie-amyloid protein elicited by non-carrier linked synthetic peptide immunogens. *J Mol Recognit*, *4*(2-3), 85-91.

[42] Ikegami, Y., Ito, M., Isomura, H., Momotani, E., Sasaki, K., Muramatsu, Y., Ishiguro, N. & Shinagawa, M. (1991). Pre-clinical and clinical diagnosis of scrapie by detection of PrP protein in tissues of sheep. *Vet Rec*, *128*(12), 271-5.

[43] Hashimoto, K., Mannen, T. & Nukina, N. (1992). Immunohistochemical study of kuru plaques using antibodies against synthetic prion protein peptides. *Acta Neuropathol*, 83(6), 613-7.

[44] Kirkwood, J. K., Wells, G. A., Cunningham, A. A., Jackson, S. I., Scott, A. C., Dawson, M. & Wilesmith, J. W. (1992). Scrapie-like encephalopathy in a greater kudu (Tragelaphus strepsiceros) which had not been fed ruminant-derived protein. *Vet Rec*,*130*(17), 365-7.

[45] Onodera, T., Ikeda, T., Muramatsu, Y. & Shinagawa, M. (1993). Isolation of scrapie agent from the placenta of sheep with natural scrapie in Japan. *Microbiol Immunol*, *37*(4), 311-6.

[46] Guiroy, D. C., Marsh, R. F., Yanagihara, R. & Gajdusek, D. C. (1993). Immunolocalization of scrapie amyloid in non-congophilic, non-birefringent deposits in golden Syrian hamsters with experimental transmissible mink encephalopathy. *Neurosci Lett*, *155*(1), 112-5.

[47] Groschup, M. H. & Pfaff, E. (1993). Studies on a species-specific epitope in murine, ovine and bovine prion protein. *J Gen Virol*, *74*(Pt 7), 1451-6.

[48] Miller, J. M., Jenny, A. L., Taylor, W. D., Marsh, R. F., Rubenstein, R. & Race, R. E. (1993). Immunohistochemical detection of prion protein in sheep with scrapie. *J Vet Diagn Invest* , *5*(3), 309-16.

[49] Groschup, M. H., Langeveld, J. & Pfaff, E. (1994). The major species specific epitope in prion proteins of ruminants. *Arch Virol*, *136*(3-4), 423-31.

[50] Xi, Y. G., Cardone, F. & Pocchiari, M. (1994). Detection of proteinase-resistant protein (PrP) in small brain tissue samples from Creutzfeldt-Jakob disease patients. *J Neurol Sci* ,*124*(2), 171-3.

[51] Schmerr, M. J., Goodwin, K. R. & Cutlip, R. C. (1994). Capillary electrophoresis of the scrapie prion protein from sheep brain. *J Chromatogr A*, *680*(2), 447-53.

[52] Yokoyama, T., Kimura, K., Tagawa, Y. & Yuasa, N. (1995). Preparation and characterization of antibodies against mouse prion protein (PrP) peptides. *Clin Diagn Lab Immunol*, *2*(2), 172-6.

[53] Yokoyama, T., Itohara, S. & Yuasa, N. (1996). Detection of species specific epitopes of mouse and hamster prion proteins (PrPs) by anti-peptide antibodies. *Arch Virol*, *141* (3-4), 763-9.

[54] Madec, J. Y., Vanier, A., Dorier, A., Bernillon, J., Belli, P. & Baron, T. (1997). Biochemical properties of protease resistant prion protein PrP^{Sc} in natural sheep scrapie. *Arch Virol*, *142*(8), 1603-12.

[55] Loftus, B. & Rogers, M. (1997). Characterization of a prion protein (PrP) gene from rabbit; a species with apparent resistance to infection by prions. *Gene*, *184*(2), 215-9.

[56] Groschup, M. H., Harmeyer, S. & Pfaff, E. (1997). Antigenic features of prion proteins of sheep and of other mammalian species. *J Immunol Methods*, *207*(1), 89-101.

[57] Komar, A. A., Lesnik, T., Cullin, C., Guillemet, E., Ehrlich, R. & Reiss C. (1997). Differential resistance to proteinase K digestion of the yeast prion-like (Ure2p) protein synthesized in vitro in wheat germ extract and rabbit reticulocyte lysate cell-free translation systems. *FEBS Lett*, *415*(1), 6-10.

[58] Takahashi, H., Takahashi, R. H., Hasegawa, H., Horiuchi, M., Shinagawa, M., Yokoyama, T., Kimura, K., Haritani, M., Kurata, T. & Nagashima, K. (1999). Characterization of antibodies raised against bovine-PrP-peptides. *J Neurovirol*, *5*(3), 300-7.

[59] Garssen, G. J., Van Keulen, L. J., Farquhar, C. F., Smits, M. A., Jacobs, J. G., Bossers, A., Meloen, R. H. & Langeveld, J. P. (2000). Applicability of three anti-PrP peptide sera including staining of tonsils and brainstem of sheep with scrapie. *Microsc Res Tech*, *50*(1), 32-9.

[60] Zhao, X., Dong, X. & Zhou, W. (2000). [Preparation of polyclonal antibody to human prion protein using the expressed GST-PrP fusion protein as antigen]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, *14*(2), 131-3.

[61] Kelker, M., Kim, C., Chueh, P. J., Guimont, R., Morré, D. M. & Morré, D. J. (2001). Cancer isoform of a tumor-associated cell surface NADH oxidase (tNOX) has properties of a prion. *Biochemistry*, *40*(25), 7351-4.

[62] Vol'pina, O. M., Zhmak, M. N., Obozhaia, M. B., Titova, M. A., Koroev, D. O., Volkova, T. D., Egorov, A. A. & Ivanov, V. T. (2001). [Antibodies against synthetic

fragments of the prion protein for the diagnosis of bovine spongiform encephalopathy]. *Bioorg Khim*, *27*(5), 352-8.

[63] Bencsik, A., Lezmi, S., Hunsmann, G. & Baron, T. (2001). Close vicinity of PrP expressing cells (FDC) with noradrenergic fibers in healthy sheep spleen. *Dev Immunol*, *8*(3-4), *235*-41.

[64] Li, Y. M., Tian, B., Zhang, B. Y. & Dong, X. P. (2001). [Diagnosis of bovine spongiform encephalopathy and scrapie by Western blot]. *Sheng Wu Gong Cheng Xue Bao*, *17*(5), 494-7.

[65] Laude, H., Vilette, D., Le Dur, A., Archer, F., Soulier, S., Besnard, N., Essalmani, R. & Vilotte, J. L. (2002). New in vivo and ex vivo models for the experimental study of sheep scrapie: development and perspectives. *C R Biol*, *325*(1), 49-57.

[66] Takekida, K., Kikuchi, Y., Yamazaki, T., Kakeya, T., Takatori, K., Tanamoto, K., Sawada, J. & Tanimura, A. (2002). [Study on the detection of prion protein in food products by a competitive enzyme-linked immunosorbent assay]. *Shokuhin Eiseigaku Zasshi*, *43*(3), 173-7.

[67] Jackman, R. & Schmerr, M. J. (2003). Analysis of the performance of antibody capture methods using fluorescent peptides with capillary zone electrophoresis with laser-induced fluorescence. *Electrophoresis*, *24*(5), 892-6.

[68] Gilch, S., Wopfner, F., Renner-Müller, I., Kremmer, E., Bauer, C., Wolf, E., Brem, G., Groschup, M. H. & Schätzl, H. M. (2003). Polyclonal anti-PrP auto-antibodies induced with dimeric PrP interfere efficiently with PrPSc propagation in prion-infected cells. *J Biol Chem*, *278*(20), 18524-31.

[69] Brun, A., Castilla, J., Ramírez, M. A., Prager, K., Parra, B., Salguero, F. J., Shiveral, D., Sánchez, C., Sánchez-Vizcaíno, J. M., Douglas, A. & Torres, J. M. (2004). Proteinase K enhanced immunoreactivity of the prion protein-specific monoclonal antibody 2A11. *Neurosci Res*, *48*(1), 75-83.

[70] Sachsamanoglou, M., Paspaltsis, I., Petrakis, S., Verghese-Nikolakaki, S., Panagiotidis, C. H., Voigtlander, T., Budka, H., Langeveld, J. P. & Sklaviadis, T. (2004). Antigenic profile of human recombinant PrP: generation and characterization of a versatile polyclonal antiserum. *J Neuroimmunol*, *146*(1-2), 22-32.

[71] Senator, A., Rachidi, W., Lehmann, S., Favier, A. & Benboubetra, M. (2004). Prion protein protects against DNA damage induced by paraquat in cultured cells. *Free Radic Biol Med*, *37*(8), 1224-30.

[72] Golańska, E., Hułas-Bigoszewska, K., Sikorska, B. & Liberski, P. P. (2005). [Analyses of 14-3-3 protein in the cerebrospinal fluid in Creutzfeldt-Jakob disease. Preliminary report]. *Neurol Neurochir Pol*, *39*(5), 358-65.

[73] Dupiereux, I., Zorzi, W., Rachidi, W., Zorzi, D., Pierard, O., Lhereux, B., Heinen, E. & Elmoualij, B. (2006). Study on the toxic mechanism of prion protein peptide 106-126 in neuronal and non neuronal cells. *J Neurosci Res*, *84*(3), 637-46.

[74] Biswas, S., Langeveld, J. P., Tipper, D. & Lu, S. (2006). Intracellular accumulation of a 46 kDa species of mouse prion protein as a result of loss of glycosylation in cultured mammalian cells. *Biochem Biophys Res Commun*, *349*(1), 153-61.

[75] Gao, C., Lei, Y. J., Han, J., Shi, Q., Chen, L., Guo, Y., Gao, Y. J., Chen, J. M., Jiang, H. Y., Zhou, W. & Dong, X. P. (2006). Recombinant neural protein PrP can bind with both recombinant and native apolipoprotein E in vitro. *Acta Biochim Biophys Sin (Shanghai)*, *38*(9), 593-601.

[76] Kocisko, D. A & Caughey, B. (2006). Searching for anti-prion compounds: cell-based high-throughput in vitro assays and animal testing strategies. *Methods Enzymol*, *412*, 223-34.

[77] Xiao, X. L., Han, J., Zhang, L., Chen, L., Zhang, J., Chen, X. L., Zhou, W., Jiang, H. Y., Zhang, B. Y., Liu, Y. & Dong, X. P. (2006). Protein expression of human neuron-specific enolase and its antiserum preparation. *Nan Fang Yi Ke Da Xue Xue Bao*, *26*(11), 1543-7.

[78] Oboznaya, M. B., Gilch, S., Titova, M. A., Koroev, D. O., Volkova, T. D., Volpina, O. M. & Schätzl, H. M. (2007). Antibodies to a nonconjugated prion protein peptide 95-123 interfere with PrP^{Sc} propagation in prion-infected cells. *Cell Mol Neurobiol*, *27*(3), 271-84.

[79] Handisurya, A., Gilch, S., Winter, D., Shafti-Keramat, S., Maurer, D., Schätzl H. M. & Kirnbauer, R. (2007). Vaccination with prion peptide-displaying papillomavirus-like particles induces autoantibodies to normal prion protein that interfere with pathologic prion protein production in infected cells. *FEBS J*, *274*(7), 1747-58.

[80] Bastian, F. O., Sanders, D. E., Forbes, W. A., Hagius, S. D., Walker, J. V., Henk, W. G., Enright, F. M. & Elzer, P. H. (2007). Spiroplasma spp. from transmissible spongiform encephalopathy brains or ticks induce spongiform encephalopathy in ruminants. *J Med Microbiol*, *56*(Pt 9), 1235-42.

[81] Dong, C. F., Wang, X. F., An, R., Chen, J. M., Shan, B., Han, L., Lei, Y. J., Han, J. & Dong, X. P. (2007). [Interaction analysis between various PrP fusion proteins and the tubulin in vitro]. *Bing Du Xue Bao*, *23*(1), 28-32.

[82] Courageot, M. P., Daude, N., Nonno, R., Paquet, S., Di Bari, M. A., Le Dur, A., Chapuis, J., Hill, A. F., Agrimi, U., Laude, H. & Vilette, D. (2008). A cell line infectible by prion strains from different species. *J Gen Virol*, *89*(Pt 1), 341-7.

[83] Sakudo, A., Nakamura, I., Tsuji, S. & Ikuta, K. (2008). GPI-anchorless human prion protein is secreted and glycosylated but lacks superoxide dismutase activity. *Int J Mol Med*, *21*(2), 217-22.

[84] Shin, W., Lee, B., Hong, S., Ryou, C. & Kwon, M. Cloning and expression of a prion protein (PrP) gene from Korean bovine (Bos taurus coreanae) and production of rabbit anti-bovine PrP antibody. *Biotechnol Lett*, *30*(10), 1705-11.

[85] Lawson, V. A., Vella, L. J., Stewart, J. D., Sharples, R. A., Klemm, H., Machalek, D. M., Masters, C. L., Cappai, R., Collins, S. J. & Hill, A. F. (2008). Mouse-adapted sporadic human Creutzfeldt-Jakob disease prions propagate in cell culture. *Int J Biochem Cell Biol*, *40*(12), 2793-801.

[86] Hanoux, V., Wijkhuisen, A., Alexandrenne, C., Créminon, C., Boquet, D. & Couraud, J. Y. (2009). Polyclonal anti-idiotypic antibodies which mimic an epitope of the human prion protein. *Mol Immunol*, *46*(6), 1076-83.

[87] Tang, H., Fu, Y., Cui, Y., He, Y., Zeng, X., Ploplis, V. A., Castellino, F. J. & Luo, Y. (2009). Fibrinogen has chaperone-like activity. *Biochem Biophys Res Commun*, *378*(3), 662-7.

[88] Ishiguro, N., Inoshima, Y., Sassa, Y. & Takahashi, T. (2009). Molecular characterization of chicken prion proteins by C-terminal-specific monoclonal antibodies. *Vet Immunol Immunopathol*, *128*(4), 402-6.

[89] Fernandez-Funez, P., Zhang, Y., Casas-Tinto, S., Xiao, X., Zou, W. Q. & Rincon-Limas, D. E. (2010). Sequence-dependent prion protein misfolding and neurotoxicity. *J Biol Chem*, *285*(47), 36897-908.

[90] Bitel, C. L., Feng, Y., Souayah, N. & Frederikse, P. H. (2010). Increased expression and local accumulation of the prion protein, Alzheimer Aβ peptides, superoxide dismutase 1, and nitric oxide synthases 1 & 2 in muscle in a rabbit model of diabetes. *BMC Physiol*, 2010 Sep 6,10:18. doi: 10.1186/1472-6793-10-18.

[91] Zocche Soprana, H., Canes Souza, L., Debbas, V. & Martins Laurindo, F. R. (2011). Cellular prion protein (PrP^C) and superoxide dismutase (SOD) in vascular cells under oxidative stress. *Exp Toxicol Pathol*, *63*(3), 229-36.

[92] Mays, C. E., Yeom, J., Kang, H. E., Bian, J., Khaychuk, V., Kim, Y., Bartz, J. C., Telling, G. C. & Ryou, C. (2011). In vitro amplification of misfolded prion protein using lysate of cultured cells. *PLoS One*, *6*(3), e18047.

[93] Julien, O., Chatterjee, S., Bjorndahl, T. C., Sweeting, B., Acharya, S., Semenchenko, V., Chakrabartty, A., Pai, E. F., Wishart, D. S., Sykes, B. D. & Cashman, N. R. (2011). Relative and regional stabilities of the hamster, mouse, rabbit, and bovine prion proteins toward urea unfolding assessed by nuclear magnetic resonance and circular dichroism spectroscopies. *Biochemistry*, *50*(35), 7536-45.

[94] Fernandez-Funez, P., Zhang, Y., Sanchez-Garcia, J., Jensen, K., Zou, W. Q. & Rincon-Limas, D. E. (2011). Pulling rabbits to reveal the secrets of the prion protein.*Commun Integr Biol*, *4*(3), 262-6.

[95] Kim, H. J., Tark, D. S., Lee, Y. H., Kim, M. J., Lee, W. Y., Cho, I. S., Sohn, H. J. & Yokoyama, T. (2012). Establishment of a cell line persistently infected with chronic wasting disease prions. *J Vet Med Sci*, *74*(10), 1377-80.

[96] Vidal, E., Fernández-Borges, N., Pintado, B., Ordóñez, M., Márquez, M., Fondevila, D., Torres, J. M., Pumarola, M. & Castilla, J. (2013). Bovine spongiform encephalopathy induces misfolding of alleged prion-resistant species cellular prion protein without altering its pathobiological features. *J Neurosci*, *33*(18), 7778-86.

[97] Wang, Y., Zhao, S., Bai, L., Fan, J. & Liu, E. (2013). Expression systems and species used for transgenic animal bioreactors. *Biomed Res Int*, 2013, 580463. doi: 10.1155/ 2013/580463.

[98] Wang, H., Yang, L., Kouadir, M., Tan, R., Wu, W., Zou, H., Wang, J., Khan, S. H., Li, D., Zhou, X., Yin, X., Wang, Y. & Zhao, D. (2013). Expression and distribution of laminin receptor precursor/laminin receptor in rabbit tissues. *J Mol Neurosci*, 2013, May 30. [Epub ahead of print]

[99] Sweeting, B., Brown, E., Khan, M. Q., Chakrabartty, A. & Pai, E. F. (2013). N-terminal helix-cap in α-helix 2 modulates β-state misfolding in rabbit and hamster prion proteins. *PLoS One*, *8*(5), e63047.

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter III

The Effect of Reduced Dietary Consistency on the Fiber Properties of Rabbit Jaw Muscles

Thorsten Grünheid*
Division of Orthodontics, School of Dentistry
University of Minnesota, Moos Health Science Tower
Minneapolis, MN

Abstract

The dynamic nature of muscle fibers enables them to adapt to altered functional requirements by changing their myosin heavy chain (MyHC) isoform and cross-sectional area. Although these changes may occur under various conditions, muscular activity plays an essential role in modulating the phenotypic properties of muscle fibers. In jaw muscles, muscular activity is influenced by the consistency of the available food. It has been shown that the continuous intake of a liquid diet, which eliminates masticatory effort, reduces muscular activity and, in turn, the functional capacity of jaw muscles. However, little is known about how jaw muscles respond to a reduction in dietary consistency within the normal range of compressive strengths of foods. Therefore, the present study investigated the effect of the long-term intake of such a diet on the MyHC composition and the fiber cross-sectional area of jaw muscles in the rabbit.

Male juvenile rabbits were randomly divided into two groups, which were raised on diets of different consistency from weaning to puberty. The experimental group was fed pellets requiring significantly reduced peak loadings, and thus lower level of jaw-muscle activity, to break the pellet in comparison with the standard pellets fed to the control group. At puberty, the MyHC composition and the corresponding cross-sectional area of fibers in the superficial masseter, superficial temporalis, medial and lateral pterygoid, and digastric muscles of both groups were determined using immunohistochemistry.

The proportion and cross-sectional area of fibers co-expressing MyHC-I and MyHC-cardiac alpha were significantly smaller in the masseter muscles of the animals that had

* Phone: 612-625-5110; Fax: 612-626-2571; E-mail: tgruenhe@umn.edu.

been fed the soft pellets than in those of the controls. In contrast, the proportions and cross-sectional areas of the various fiber types in the other jaw muscles did not differ significantly between the groups.

These findings suggest that the long-term reduction in the dietary consistency contributes to selective disuse resulting in structural adaptation of the masseter muscle, the jaw-closing muscle primarily responsible for generating occlusal force during chewing, reflected in decreases in the proportion and cross-sectional area of its slow fibers. At the same time, it appears that the reduced activity during mastication of a diet with the consistency of soft foods normally eaten by rabbits is sufficient to prevent disuse atrophy in the other jaw muscles.

Overall, the rabbit masticatory system seems to be relatively rigid, manifesting few diet-specific changes in the fiber properties of jaw muscles.

Introduction

Jaw Muscles

From a classical anatomical perspective, the jaw muscles are divided into elevator and depressor groups. The elevator group consists of the masseter, temporalis, and medial pterygoid muscles, while the depressor group consists of the geniohyoid, mylohyoid, and digastric muscles. The lateral pterygoid muscle completes the system. Because its two heads have different actions, this muscle cannot be regarded exclusively as elevator or depressor (McNamara, 1973; Juniper, 1981).

Jaw muscles, like all skeletal muscles, are capable of powerful contractions by virtue of the regular organization of their contractile proteins. The cellular units of a skeletal muscle are the muscle fibers, long cylindrical cells with multiple nuclei. Each muscle fiber contains many myofibrils that run parallel to its length. The myofibrils are composed of sarcomeres, arranged end to end. Sarcomeres, the contractile units of a skeletal muscle fiber, consist of two types of myofilament organized into regular arrays with a partially overlapping structure. The thin filaments contain mainly actin, whereas the thick filaments contain mainly myosin. Myosin consists of two intertwined heavy peptide chains (Gazith et al., 1970) and four light peptide chains (Lowey and Risby, 1971). Because it contains the ATPase, which determines the unloaded shortening velocity, the myosin heavy chain (MyHC) is primarily responsible for the contraction velocity of the muscle fiber (Staron, 1991; Larsson and Moss, 1993).

On the basis of the MyHC isoform they contain, skeletal muscle fibers have been classified into slow (type I) and fast (type II) fibers, with subclassification of fast fibers including types IIA, IIB, and IIX (*e.g.*, Schiaffino and Reggiani, 1994). Motor unit size, fiber diameter, contraction velocity, and tetanic force increase successively by fiber type in the order of I, IIA, IIX, and IIB (Sciote and Kentish, 1996; Sciote et al., 2003), while fatigue resistance decreases in the same order (Bottinelli et al., 1996).

Jaw muscles are notably different from other skeletal muscles. For instance, healthy adult jaw-closing muscles express MyHC-fetal and MyHC-cardiac alpha, MyHC isoforms typically present in developing and cardiac muscle, respectively, in addition to types I, IIA, and IIX (Sciote et al., 1994). The contraction velocity of MyHC-fetal fibers seems to be low (D'Antona et al., 2003); the contraction velocity of MyHC-cardiac alpha fibers lies between those of MyHC-I and -IIA fibers (Kwa et al., 1995; Sciote and Kentish, 1996; Galler et al.,

2002). Many of the jaw-muscle fibers are hybrids, expressing two or more MyHC isoforms in various combinations (Bredman et al., 1991; Monemi et al., 1996, 1998; Korfage et al., 2005a, 2005b). These hybrid fibers have contractile properties intermediate to those of pure fibers. For instance, hybrid fibers that express both MyHC-I and -IIA are faster than pure MyHC-I fibers but slower than pure MyHC-IIA fibers. The diversity of MyHC isoforms and the large number of hybrid fibers present in jaw muscles provides a mechanism that allows very fine gradation of contraction velocity (Pette, 2002).

Muscle Adaptation

The ability to adapt to varying requirements is an archetypical characteristic of tissues, which modify their properties in accordance with the mechanical environment. Skeletal muscles are able to change their anatomical characteristics, such as size and fiber types, to adapt to altered functional requirements (Adams et al., 1993; Pette, 2002). For instance, resistance training by means of repeated isometric contraction and relaxation causes an increase in muscle thickness and enhances muscular strength (Hather et al., 1991; Staron et al., 1994). In contrast, detraining or immobilization of a muscle leads to disuse atrophy.

The structural adaptive growth is associated with changes in individual fibers, which can adapt to varying requirements by changing from one fiber type into another (Adams et al., 1993; Pette and Staron, 1997). Muscle fibers can change their phenotype by switching different MyHC isoform genes on or off. In mammals, this conversion of fiber types follows a strict order: MyHC-I → -IIA → -IIX → -IIB or *vice versa* (Schiaffino and Reggiani, 1994). Although fiber-type transitions can occur under the influence of various factors and conditions (Grünheid et al., 2009), muscular activity plays an essential role in modulating the phenotypic properties of muscle fibers (Roy et al., 1991; Kernell et al., 1998; Gorassini et al., 1999). In general, increased muscular activity elicits transition towards slower, more fatigue-resistant fiber types and enlargement of fiber cross-sectional area, whereas reduced muscular activity induces transition towards faster, more fatigable fiber types and a decrease in fiber cross-sectional area (Thomason and Booth, 1990; Adams et al., 1993; Caiozzo et al., 1996; Jarvis et al., 1996; Tipton et al., 1996; Pette and Staron, 1997; Grossman et al., 1998; Pette, 2001).

The fiber types of jaw muscles can be determined using monoclonal antibodies raised against various isoforms of purified MyHC (Bredman et al., 1991, 1992; Korfage and van Eijden, 2003). The cross-sectional areas of individual fibers can be quantified on microphotographs of transverse sections of the muscle. When subsequent sections are incubated with antibodies directed against various MyHC isoforms, the cross-sectional area of a fiber can be related to its MyHC isoform content (Korfage et al., 2000).

Dietary Consistency

The activity of jaw muscles can be altered experimentally by changing the consistency of the available food. For instance, the continuous intake of a soft diet, which requires less masticatory effort, has been shown to reduce the activity and, in turn, the functional capacity

of jaw muscles (Kiliaridis and Shyu, 1988; Liu et al., 1998). Although this experimental approach has been used in various species, information on the associated changes in MyHC composition and fiber cross-sectional area in the rabbit jaw muscles is scarce as previous investigations (Negoro et al., 2001; Langenbach et al., 2003; Kitagawa et al., 2004) were limited to the masseter muscle. Therefore, an experiment was performed to investigate the effect of a reduction in the dietary consistency on the MyHC composition and the fiber cross-sectional area of the rabbit masseter, temporalis, medial and lateral pterygoid, and digastric muscles.

Animal Experiment

Animals

Sixteen male juvenile New Zealand White rabbits (*Oryctolagus cuniculus*, Harlan, Horst, the Netherlands) were used. When obtained, they were 6 weeks old and weighed 1,402 ± 189 g. The animals were housed individually in metal cages with perforated plastic floors, kept in a climate controlled room (22.0 ± 0.9°C) with a 12-hour light-dark cycle, and fed a commercially manufactured pelleted diet (Arie Blok, Woerden, the Netherlands) and water *ad libitum*. All animals were kept under identical conditions before they were randomly divided into two equal-sized groups at the age of 8 weeks.

Experimental Design

The experimental group was fed a diet of soft pellets requiring significantly reduced peak loadings (10 N/cm^2), and thus level of jaw-muscle contractions, to break the pellet in comparison with the standard pellets (120 N/cm^2) fed to the control group. The pellets did not differ in size or nutritional value. No environmental enrichment was provided in order to prevent the animals from gnawing. Body weight and physical condition were checked weekly to monitor growth and health of the animals. At the age of 20 weeks, the animals were sedated with 0.6 ml/kg body weight of a 1:3 mixture of xylazine (Sedazine, AST Farma, Oudewater, the Netherlands) and ketamine (Ketamine, Alfasan, Woerden, the Netherlands), and killed by an intravenous overdose of sodium pentobarbital (Euthesate, Ceva Sante Animale, Naaldwijk, the Netherlands). The experiment had been approved by the local Animal Ethics Committee and was performed in accordance with the animal care and welfare guidelines of the National Institute of Health.

Analysis of Muscle Fibers

The fibers of the superficial masseter, superficial temporalis, medial and lateral pterygoid, and digastric muscles were analyzed using immunohistochemistry. The muscles were cut from their attachment sites with the jaws of the animals closed to ensure that the

muscles were not stretched. All muscles were obtained within 8 hours *post mortem*, rapidly frozen in liquid nitrogen-cooled isopentane, and stored at –80°C for further processing.

Serial sections (10 µm) were cut perpendicular to the main fiber direction of the muscles in a cryomicrotome (CM 1850, Leica Microsystems, Nussloch, Germany), mounted on glass slides coated with 3-aminopropyltriethoxysilane, and fixated overnight in a mixture of methanol, acetone, acetic acid, and water (35:35:5:25) at –20°C. The sections were incubated with five monoclonal antibodies raised against different MyHC isoforms: antibody 219-1D1 detected MyHC-I, antibody 249-5A4 detected MyHC-cardiac alpha, antibody 333-7H1 detected MyHC-IIA, antibody 332-3D4 detected MyHC-IIA and MyHC-IIX, and antibody 340-3B5 detected MyHC-IIA, MyHC-IIX, and MyHC-IIB (Sant'ana Pereira et al., 1995). The binding of the antibodies was visualized using the indirect unconjugated immunoperoxidase (PAP) technique with nickel-diaminobenzidine as a substrate (Hancock, 1986).

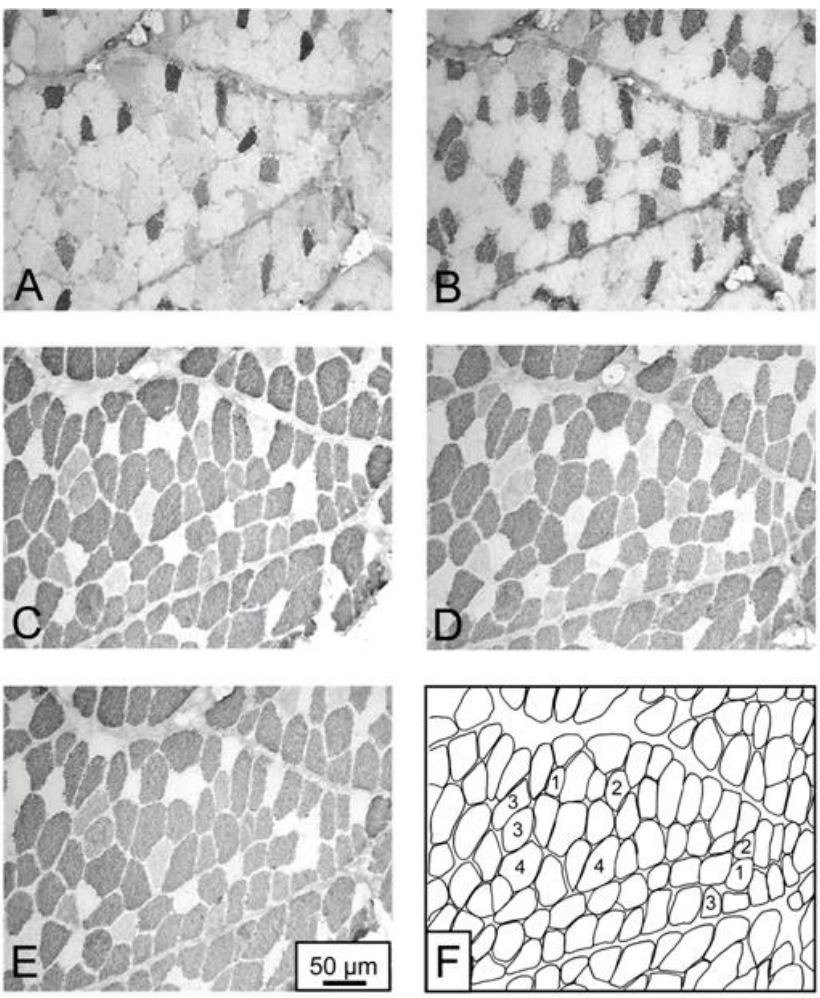


Figure 1. Example of an area of the superficial masseter muscle incubated with antibodies against myosin heavy chain (MyHC)-I (A), MyHC-cardiac alpha (B), MyHC-IIA (C), MyHC-IIA and -IIX (D), and MyHC-IIA, -IIX, and IIB isoforms (E). The drawing (F) shows some of the fiber types: (1) MyHC type I + cardiac alpha, (2) MyHC type cardiac alpha, (3) MyHC type cardiac alpha + IIA, and (4) MyHC type IIA.

The MyHC composition of muscle fibers was determined on five consecutive sections of each muscle per animal. The sections were evaluated at ×100 magnification using a light microscope equipped with a digital camera (Orthoplan, Leitz, Wetzlar, Germany). Depending on the cross-sectional area of the muscle, either two (temporalis, lateral pterygoid, and digastric), four (medial pterygoid), or six (masseter) areas of each section were evaluated. All fibers that could clearly be identified in each of the five sections were classified according to their reaction to the various antibodies (Figure 1). A total of 33,103 fibers were analyzed.

On the basis of their MyHC composition, and thus contraction velocity, the fibers were divided into three groups: fibers containing MyHC-I, MyHC-cardiac alpha, or a combination of these MyHCs were considered slow fibers (Galler et al., 2002); fibers containing MyHC-IIA, MyHC-IIX, MyHC-IIB, or a combination of these MyHCs were considered fast fibers; fibers containing a combination of the MyHC types belonging to slow and fast groups were considered intermediate fibers (Pette and Staron, 2001). The cross-sectional areas of the muscle fibers, which had been classified on the basis of the MyHCs they expressed, were then quantified by a custom-made computer program (Korfage et al., 2006a).

Statistical Analysis

Mean values and standard deviations of fiber-type proportions and fiber cross-sectional areas of the masseter, temporalis, medial and lateral pterygoid, and digastric muscles were calculated for each group of animals. Differences between the groups were tested for statistical significance, for each muscle separately, using a Mann-Whitney rank sum test after the data had been tested for normality (Kolmogorov-Smirnov test). *P*-values of less than 0.05 were considered statistically significant.

Results

All animals grew continuously throughout the experimental period. Their body weight increased from 1,930 ± 269 g at 8 weeks of age, when the change in dietary consistency was introduced, to 3,589 ± 375 g at 20 weeks of age in the experimental group, and from 1,901 ± 167 g at 8 weeks of age to 3,529 ± 183 g at 20 weeks of age in the control group. The body weight of the animals did not differ significantly between the groups at any time. The animals in the experimental group did not show any change in their masticatory pattern in response to the reduced food hardness. Mean values and standard deviations of the proportions and corresponding cross-sectional areas of the various fiber types in the jaw muscles are shown in Tables 1 and 2. No fibers expressing MyHC-IIB were detected in any of the muscles studied. Statistical testing revealed significant differences in the population of slow fibers between the groups: the fibers co-expressing MyHC-I and MyHC-cardiac alpha accounted for a smaller proportion in the masseter muscles of the experimental animals than in those of the controls. These fibers had also smaller cross-sectional areas in the experimental group than in the control group. The proportions and cross-sectional areas of the various fiber types in the other jaw muscles did not differ significantly between experimental and control animals. The relatively large standard deviations indicate substantial interindividual variation in fiber-type composition and fiber cross-sectional area in both groups.

Table 1. Fiber-type proportions in jaw muscles of rabbits raised on diets of different consistency

Fiber-type proportion (%)										
	Masseter		Temporalis		Medial pterygoid		Lateral pterygoid		Digastric	
	Soft diet	Control	Soft diet	Control	Soft diet	Control	Soft diet	Control	Soft diet	Control
Slow fibers										
I[a]	0 ± 0	0.03 ± 0.09	0.04 ± 0.10	0.07 ± 0.13	0.10 ± 0.16	0.04 ± 0.10	0.02 ± 0.06	0.05 ± 0.10	5.76 ± 3.57	7.68 ± 4.80
α[a]	9.70 ± 8.38	7.81 ± 5.61	0.43 ± 0.74	0.69 ± 1.26	14.74 ± 5.93	11.54 ± 4.9	2.58 ± 2.43	3.45 ± 2.55	0.08 ± 0.15	0.14 ± 0.20
I + α[a]	10.29 ± 4.72*	17.58 ± 5.48*	3.56 ± 3.19	5.75 ± 6.60	12.08 ± 5.67	12.57 ± 4.54	14.95 ± 6.78	17.11 ± 4.33	24.19 ± 8.67	19.09 ± 6.08
Intermediate fibers										
I + α + IIA[a]	0.94 ± 1.00	0.50 ± 0.79	1.64 ± 1.35	2.35 ± 3.22	0.29 ± 0.29	0.42 ± 0.74	0.19 ± 0.32	0.42 ± 0.67	1.68 ± 1.13	2.06 ± 1.25
I + α + IIX[a]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.99 ± 1.91	0.71 ± 0.62
I + IIA[a]	0.92 ± 1.65	0 ± 0	1.15 ± 2.99	0.67 ± 1.17	0 ± 0	0 ± 0	0.04 ± 0.08	0.03 ± 0.07	0.17 ± 0.38	0.21 ± 0.18
I + IIX[a]	0 ± 0	0 ± 0	0.03 ± 0.08	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
α + IIA[a]	17.81 ± 8.42	14.08 ± 4.63	1.78 ± 1.37	2.23 ± 1.81	12.15 ± 3.82	14.59 ± 4.76	1.58 ± 1.79	1.26 ± 0.78	1.32 ± 1.42	1.28 ± 0.83
α + IIX[a]	0.04 ± 0.12	0.27 ± 0.52	0 ± 0	0.05 ± 0.15	0.11 ± 0.31	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fast fibers										
IIA[a]	59.70 ± 9.05	57.28 ± 9.94	71.05 ± 15.61	72.18 ± 19.60	57.35 ± 6.15	56.44 ± 5.79	69.71 ± 8.16	65.67 ± 7.29	65.72 ± 8.45	67.47 ± 9.56
IIX[a]	0.60 ± 1.21	2.45 ± 3.13	20.32 ± 15.03	16.01 ± 14.57	3.18 ± 2.56	4.41 ± 5.79	10.91 ± 5.86	12.00 ± 9.44	0.09 ± 0.25	1.36 ± 3.70

Results are mean values ± standard deviations.

[a] Myosin heavy chain isoform. α, cardiac alpha.

* Statistically significant difference between groups, Mann-Whitney rank sum test $P < 0.05$.

Table 2. Cross-sectional area of the various fiber types in jaw muscles of rabbits raised on diets of different consistency

Fiber cross-sectional area (μm²)										
	Masseter		Temporalis		Medial pterygoid		Lateral pterygoid		Digastric	
	Soft diet	Control	Soft diet	Control	Soft diet	Control	Soft diet	Control	Soft diet	Control
Slow fibers										
I[a]		231 ± 0	110 ± 0	221 ± 0	1,381 ± 585	995 ± 0	587 ± 0	637 ± 42	1,142 ± 210	1,104 ± 404
α[a]	1,759 ± 263	1,624 ± 566	363 ± 114	438 ± 139	1,758 ± 405	2,040 ± 245	792 ± 342	784 ± 174	348 ± 13	500 ± 387
I + α[a]	1,085 ± 90*	1,258 ± 289*	439 ± 268	566 ± 264	1,189 ± 389	1,360 ± 288	714 ± 259	695 ± 134	1,158 ± 231	1,111 ± 377
Intermediate fibers										
I + α + IIA[a]	1,856 ± 1,166	1,614 ± 623	765 ± 773	977 ± 665	1,469 ± 655	2035 ± 463	444 ± 82	889 ± 381	1,125 ± 285	1,184 ± 399
I + α + IIX[a]									1,169 ± 135	920 ± 382
I + IIA[a]	3,677 ± 3,246		312 ± 280	723 ± 204			951 ± 864	711 ± 0	635 ± 120	923 ± 535
I + IIX[a]			488 ± 0							
α + IIA[a]	1,896 ± 485	2,019 ± 588	410 ± 228	592 ± 325	2,185 ± 485	2,518 ± 511	729 ± 290	729 ± 159	1,537 ± 365	1,220 ± 364
α + IIX[a]	1,976 ± 0	2,342 ± 265		5,287 ± 0	2,446 ± 0					
Fast fibers										
IIA[a]	4,748 ± 615	4,775 ± 1,255	1,531 ± 692	1,853 ± 621	4,297 ± 687	4,830 ± 1,035	1,483 ± 281	1,524 ± 257	2,762 ± 516	2,474 ± 813
IIX[a]	6,116 ± 4,669	6,749 ± 809	3,141 ± 1,120	3,637 ± 1,218	5,742 ± 1,695	6840 ± 1396	2,223 ± 425	1,835 ± 751	3,976 ± 0	2,026 ± 6

Results are mean values ± standard deviations.

[a] Myosin heavy chain isoform. α, cardiac alpha.

* Statistically significant difference between groups, Mann-Whitney rank sum test $P < 0.05$.

Discussion

The results of the present experiment provide a comprehensive description of the fiber properties of rabbit jaw muscles both under steady-state conditions and after a reduction in dietary consistency from weaning to puberty. The immunohistochemical determination of their MyHC composition allowed accurate characterization of the muscle fibers (Sant'ana Pereira et al., 1995), including those expressing the cardiac alpha MyHC isoform. However, it should be noted that, with the antibody panel used, it was not possible to detect hybrid fibers that co-express MyHC-IIA and MyHC-IIX. These fibers might have been misclassified as type IIA fibers.

A comparison of the proportions of the various fiber types reveals that the fiber-type composition varies among the muscles studied. For instance, the masseter and medial pterygoid muscles contain the highest percentage of slow and intermediate fibers as well as the highest percentage of hybrid fibers. It appears that these jaw closers, which are the largest muscles in the rabbit masticatory system, are well suited to perform slow, tonic movements and to produce a smooth, gradable force. In contrast, the temporalis muscle contains the highest percentage of fast fibers and the smallest percentage of hybrid fibers. These differences suggest that the fiber-type compositions of muscles within a particular muscle group differ and that the temporalis muscle is significantly faster than the other jaw closers.

Among the muscles studied, the masseter muscle contains the highest proportion of fibers expressing MyHC-cardiac alpha in combination with either MyHC-I or MyHC-IIA. The functional significance of these hybrid fibers is based on their contractile properties, which lie between those of pure fibers (Larsson and Moss, 1993; Widrick et al., 1996). For instance, hybrid fibers co-expressing MyHC-I and -cardiac alpha are faster than pure type I fibers but slower than pure type cardiac alpha fibers. Because of these properties, jaw-muscle fibers can use the cardiac alpha MyHC isoform as a "fine-tuning" link protein between the slow-type MyHC-I and the fast-type MyHC-IIA isoforms.

It has been suggested that high proportions of hybrid fibers in muscles under steady-state conditions match specific functional demands (Korfage et al., 2005b). The greater the number of different hybrid fibers, the more a continuum exists in contractile properties. In the masseter muscle, this continuum in contractile properties might contribute to a more precise modulation of mandibular position and occlusal force. Furthermore, the proportion of hybrid fibers is considered to be a reflection of the adaptive potential of a muscle because co-expression of MyHC isoforms is also a characteristic of fibers in transition from one pure fiber type to another (Stephenson, 2001; Pette, 2002). During these transitions, the fibers can change from a pure fiber type, which expresses only one MyHC isoform, into another pure fiber type via a hybrid fiber type, which expresses the old as well as the new MyHC isoform. The high proportion of hybrid fibers under steady-state conditions might, therefore, be indicative of the adaptive potential of the rabbit masseter muscle.

In addition to the differences in fiber-type composition, there is also a difference in fiber cross-sectional area among the jaw muscles. The masseter and medial pterygoid muscles have, in general, the largest cross-sectional areas of all fiber types. The only exception is the mean cross-sectional area of pure type I fibers, which is largest in the digastric muscle. The digastric muscle also has the smallest range of fiber cross-sectional areas. For instance, its pure type IIX fibers have about 3-4 times the cross-sectional areas of pure type I fibers,

whereas in the masseter muscle, which has the largest range of cross-sectional areas, pure type IIX fibers have about 25-30 times the cross-sectional areas of pure type I fibers. In general, the differences in cross-sectional area are more pronounced in jaw closers than in jaw openers.

The proportions and cross-sectional areas of the various fiber types in the jaw muscles, as well as the degree of interindividual variation in fiber-type composition and fiber cross-sectional area, are in accordance with those reported for developing rabbit jaw muscles (van Wessel et al., 2005; Korfage et al., 2006a, 2006b, 2009). A number of factors contribute to the large interindividual variation in fiber-type composition (Korfage et al., 2005b). For instance, fiber-type composition is related to age and gender, and is subject to hormonal and local, activation- and stretch-related, influences. As in the present experiment the animals were matched for age and gender in order to exclude influences of ageing and sexual dimorphism (English et al., 1999), the interindividual variation was most likely the result of other factors known to influence the phenotypic properties of jaw-muscle fibers, such as individually different masticatory patterns (Kemsley et al., 2003) or levels of testosterone (Reader et al., 2001).

The experimental reduction in dietary consistency caused significant changes in the MyHC composition and the cross-sectional area of some fibers in the masseter muscle, while those of the other jaw muscles remained unchanged. These results suggest intermuscular differences in the adaptive response of the jaw muscles to the altered functional conditions. It appears that, despite a concerted use of muscles in the masticatory system, the experimental stimulus did not affect all jaw muscles in the same way. This disparity is most likely based on the different functions of the jaw muscles during mastication. For instance, the masseter muscle elevates the mandible and generates occlusal force during the power stroke (Widmer et al., 2003) while the superficial temporalis and digastric muscles stabilize and open the jaw, respectively (Weijs et al., 1989). During chewing, it requires less muscle force to break soft food than to break hard food but the force necessary to stabilize or open the jaw is, in all likelihood, independent of the hardness of the ingested food. It appears that the reduction in dietary consistency altered only the functional loading of the muscles that are the main generators of the force necessary to crush the pellets. Therefore, only the masseter muscle adjusted its fiber-type composition and fiber cross-sectional area, while the other jaw muscles were unaffected by the change in dietary consistency.

In addition to the intermuscular differences in the adaptive response, there were differences in the adaptive response among the various fiber types in the masseter muscle. The reduction in dietary consistency induced a significant decrease in the proportion and the cross-sectional area only in fibers of the slow fiber compartment, most likely as a consequence of the frequency of their recruitment during chewing. Following the so-called “size principle” (Henneman, 1981), the motor units of jaw muscles are recruited in a strict hierarchical order (Scutter and Türker, 1998). Small motor units with predominantly slow fibers are recruited at lower force threshold levels than the larger ones with faster fibers. Mastication of pellets requires comparatively low forces. It has been shown by means of electromyography that the majority of jaw-muscle contractions during chewing in rabbits generate only 20 percent of the maximum force (Langenbach et al., 2004). In order to generate this force level, predominantly small motor units are recruited as their fibers are optimally suited for sustained contractions requiring relatively low force (Maxwell et al., 1980). It can be assumed that mainly the fibers of these motor units adapted their MyHC

composition and cross-sectional area to the reduced masticatory load. Larger motor units with faster fibers, which are only used during larger efforts for brief periods of time, are not recruited during chewing and consequently not affected by an alteration in food hardness.

The present findings are, on the whole, in agreement with those of other studies that investigated the effect of reduced masticatory function on the phenotypic properties of rabbit jaw muscles. For instance, the masseter muscles of rabbits fed a liquefied diet after puberty adapted to the reduced dietary consistency by decreasing the cross-sectional area of their slow fibers (Langenbach et al., 2003). When the animals were fed a powdered diet soon after weaning, the masseter muscles also responded with a decrease in the percentage of type I fibers and a concomitant increase in type IIA fibers (Negoro et al., 2001; Kitagawa et al., 2004), which indicates a shift in the fiber-type composition towards a higher proportion of fast fibers.

It has to be noted that, although the experimental approach of feeding a liquefied or powdered diet imposes greater differences in masticatory functional loads on experimental and control animals, it alters more than just the dietary consistency, too. Liquefied or powdered food eliminates the need for mastication (Mavropoulos et al., 2004) and changes the pattern of food uptake from incising and chewing into licking and sucking (Kitagawa et al., 2004). In contrast to this experimental approach, the present experiment used purpose-made soft pellets, which did not change the feeding behavior of the experimental animals. With regard to the difference from the consistency of the standard pellets fed to the control animals, these pellets mimicked the difference in the compressive strength between hard and soft foods normally eaten by rabbits.

Studies on other species, such as ferrets (He et al., 2004) and rats (Kiliaridis et al., 1988) found, in accordance with the present results, no significant differences in the fiber-type composition of the temporalis and digastric muscles after the normal diet had been changed to a liquefied one. In contrast, when rats were fed by gastric gavage (Sfondrini et al., 1996), the population of IIB fibers in both the temporalis and the digastric muscles increased while the population of IIA and IIX fibers decreased. It seems that the complete elimination of masticatory movements leads to severe disuse atrophy of all jaw muscles.

Adaptive responses depend on timing, duration, and intensity of a given stimulus. In the present experiment, the stimulus, *i.e.*, the reduction in dietary consistency, induced significant changes in the phenotypic properties of the less recruited masseter muscles. It seems, therefore, unlikely that the timing or the duration of the stimulus were ineligible to induce adaptive changes in the other jaw muscles. Considering the changes in fiber properties reported after complete elimination of masticatory movements, it is more likely that the stimulus was not of appropriate nature or sufficiently intensive to effectuate significant changes in the other muscles. A different type or more intensive stimulus might be required to induce changes in the phenotypic properties of the fibers in the other jaw muscles.

Conclusion

The long-term reduction in the dietary consistency within the normal range of compressive strengths of foods normally eaten by rabbits contributes to selective disuse of the muscles that are less recruited as a consequence of the lower force level necessary to crush

the food. It appears that only these muscles adapt structurally to the reduced functional load. The adaptive changes are reflected in decreases in the proportion and cross-sectional area of slow fibers in the affected muscles. At the same time, it seems that the reduced masticatory muscular activity is sufficient to prevent disuse atrophy in the other jaw muscles. Overall, the rabbit masticatory system appears to be relatively rigid, manifesting few diet-specific changes in the fiber properties of jaw muscles.

References

Adams GR, Hather BM, Baldwin KM, Dudley GA (1993). Skeletal muscle myosin heavy chain composition and resistance training. *J. Appl. Physiol.* 74:911–915.

Bottinelli R, Canepari M, Pellegrino MA, Reggiani C (1996). Force-velocity properties of human skeletal muscle fibers: myosin heavy chain isoform and temperature dependence. *J. Physiol.* 495:573–586.

Bredman JJ, Wessels A, Weijs WA, Korfage JAM, Soffers CA, Moorman AFM (1991). Demonstration of 'cardiac-specific' myosin heavy chain in masticatory muscles of human and rabbit. *Histochem. J.* 23:160–170.

Bredman JJ, Weijs WA, Korfage JAM, Brugman P, Moorman AFM (1992). Myosin heavy chain expression in rabbit masseter muscle during postnatal development. *J. Anat.* 180:263–274.

Caiozzo VJ, Haddad F, Baker MJ, Herrick RE, Prietto N, Baldwin KM (1996). Microgravity-induced transformations of myosin isoforms and contractile properties of skeletal muscle. *J. Appl. Physiol.* 81:123–132.

D'Antona G, Pellegrino MA, Adami R, Rossi R, Carlizzi CN, Canepari M, Saltin B, Bottinelli R (2003). The effect of ageing and immobilization on structure and function of human skeletal muscle fibres. *J. Physiol.* 552:499–511.

English AW, Eason J, Schwartz G, Shirley A, Carrasco DI (1999). Sexual dimorphism in the rabbit masseter muscle: myosin heavy chain composition of neuromuscular compartments. *Cells Tissues Organs* 164:179–191.

Galler S, Puchert E, Gohlsch B, Schmid D, Pette D (2002). Kinetic properties of cardiac myosin heavy chain isoforms in rat. *Pflugers Arch* 445:218–223.

Gazith J, Himmelfarb S, Harrington WF (1970). Studies on the subunit structure of myosin. *J. Biol. Chem.* 245:15–22.

Gorassini M, Bennett DJ, Kiehn O, Eken T, Hultborn H (1999). Activation patterns of hindlimb motor units in the awake rat and their relation to motoneuron intrinsic properties. *J. Neurophysiol.* 82:709–717.

Grossman EJ, Roy RR, Talmadge RJ, Zhong H, Edgerton VR (1998). Effects of inactivity on myosin heavy chain composition and size of rat soleus fibres. *Muscle Nerve* 21:375–389.

Grünheid T, Langenbach GEJ, Korfage JAM, Zentner A, van Eijden TMGJ (2009). The adaptive response of jaw muscles to varying functional demands. *Eur. J. Orthod.* 31:596–612.

Hancock MB (1986). Two-color immunoperoxidase staining: visualization of anatomic relationships between immunoreactive neural elements. *Am. J. Anat.* 175:343–352.

Hather BM, Tesch PA, Buchanan P, Dudley GA (1991). Influence of eccentric actions on skeletal muscle adaptations to resistance training. *Acta Physiol. Scand.* 143:177–185.

He T, Olsson S, Daugaard JR, Kiliaridis S (2004). Functional influence of masticatory muscles on the fiber characteristics and capillary distribution in growing ferrets (*Mustela putonusfuro*)–a histochemical analysis. *Arch. Oral Biol.* 49:983–989.

Henneman E (1981). Recruitment of motoneurons: the size principle. *Prog. Clin. Neurophysiol.* 9:26–60.

Jarvis JC, Mokrusch T, Kwende MMN, Sutherland H, Salmons S (1996). Fast to slow transformation in stimulated rat muscle. *Muscle Nerve* 19:1469–1475.

Juniper (1981). The superior pterygoid muscle? *Br. J. Oral Surg.* 19:121–128.

Kemsley EK, Defernez M, Sprunt JC, Smith AC (2003). Electromyographic responses to prescribed mastication. *J. Electromyogr Kinesiol.* 13:197–207.

Kernell D, Hensbergen E, Lind A, Eerbeek O (1998). Relation between fibre composition and daily duration of spontaneous activity in ankle muscles of the cat. *Arch. Ital. Biol.* 136:191–203.

Kiliaridis S, Engström C, Thilander B (1988). Histochemical analysis of masticatory muscle in the growing rat after prolonged alteration in the consistency of the diet. *Arch. Oral. Biol.* 33:187–193.

Kiliaridis S, Shyu BC (1988). Isometric muscle tension generated by masseter stimulation after prolonged alteration of the consistency of the diet fed to growing rats. *Arch. Oral. Biol.* 33:467–472.

Kitagawa Y, Mitera K, Ogasawara T, Nojyo Y, Miyauchi K, Sano K (2004). Alterations in enzyme histochemical characteristics of the masseter muscle caused by long-term soft diet in growing rabbits. *Oral Dis.* 10:271–276.

Korfage JAM, Brugman P, van Eijden TMGJ (2000). Intermuscular and intramuscular differences in myosin heavy chain composition of the human masticatory muscles. *J. Neurol. Sci.* 178:95–106.

Korfage JAM, Schueler YT, Brugman P, van Eijden TMGJ (2001). Differences in myosin heavy-chain composition between human jaw-closing muscles and supra- and infrahyoid muscles. *Arch. Oral Biol.* 46:821–827.

Korfage JAM, van Eijden TMGJ (2003). Myosin heavy chain composition in human masticatory muscles by immunohistochemistry and gel electrophoresis. *J. Histochem. Cytochem.* 51:113–119.

Korfage JAM, Koolstra JH, Langenbach GEJ, van Eijden TMGJ (2005a). Fiber-type composition of the human jaw muscles–(part 1) origin and functional significance of fiber-type diversity. *J. Dent Res.* 84:774–783.

Korfage JAM, Koolstra JH, Langenbach GEJ, van Eijden TMGJ (2005b). Fiber-type composition of the human jaw muscles–(part 2) role of hybrid fibers and factors responsible for inter-individual variation. *J. Dent Res.* 84:784–793.

Korfage JAM, van Wessel T, Langenbach GEJ, Ay F, van Eijden TMGJ (2006a). Postnatal transitions in myosin heavy chain isoforms of the rabbit superficial masseter and digastric muscle. *J. Anat.* 208:743–751.

Korfage JAM, van Wessel T, Langenbach GEJ, van Eijden TMGJ (2006b). Heterogeneous postnatal transitions in myosin heavy chain isoforms within the rabbit temporalis muscle. *Anat. Rec. A. Discov. Mol. Cell Evol. Biol.* 288:1095–1104.

Korfage JAM, Helmers R, de Goüyon Matignon M, van Wessel T, Langenbach GEJ, van Eijden TMGJ (2009). Postnatal development of fiber type composition in rabbit jaw and leg muscles. *Cells Tissues Organs* 190:42–52.

Kwa SH, Weijs WA, Jüch PJ (1995). Contraction characteristics and myosin heavy chain composition of rabbit masseter motor units. *J. Neurophysiol.* 73:538–549.

Langenbach GEJ, van de Pavert S, Savalle WPM, Korfage JAM, van Eijden TMGJ (2003). Influence of food consistency on the rabbit masseter muscle fibers. *Eur. J. Oral Sci.* 111:81–84.

Langenbach GEJ, van Wessel T, Brugman P, van Eijden TMGJ (2004). Variation in daily masticatory muscle activity in the rabbit. *J. Dent Res.* 83:55–59.

Larsson L, Moss RL (1993). Maximum velocity of shortening in relation to myosin isoform composition in single fibres from human skeletal muscles. *J. Physiol.* 472:595–614.

Liu ZJ, Ikeda K, Harada S, Kasahara Y, Ito G (1998). Functional properties of jaw and tongue muscles in rats fed a liquid diet after being weaned. *J. Dent Res.* 77:366–376.

Lowey S, Risby D (1971). Light chains from fast and slow muscle myosins. *Nature* 234:81–85.

Mavropoulos A, Kiliaridis S, Bresin A, Ammann P (2004). Effect of different masticatory functional and mechanical demands on the structural adaptation of the mandibular alveolar bone in young growing rats. *Bone* 35:191–197.

Maxwell LC, McNamara JA Jr, Carlson DS, Faulkner JA (1980). Histochemistry of fibers of masseter and temporalis muscles of edentulous monkeys *Macaca mulatta*. *Arch. Oral Biol.* 25:87–93.

McNamara JA Jr (1973). The independent functions of the two heads of the lateral pterygoid muscle. *Am. J. Anat.* 138:197–205.

Monemi M, Eriksson P-O, Dubail I, Butler-Browne GS, Thornell L-E (1996). Fetal myosin heavy chain increases in the human masseter muscle during aging. *FEBS Lett.* 386:87–90.

Monemi M, Eriksson P-O, Eriksson A, Thornell L-E (1998). Adverse changes in fiber-type composition of the human masseter versus biceps brachii muscle during aging. *J. Neurol. Sci.* 154:35–48.

Negoro T, Ito K, Morita T, Hiraba K, Mizutani M, Ohno N, Goto S (2001). Histochemical study of rabbit masseter muscle: The effect of the alteration of food on the muscle fibers. *Oral Med. Pathol.* 6:65–71.

Pette D (2001). Plasticity in skeletal, cardiac, and smooth muscle. Historical perspectives: Plasticity of mammalian skeletal muscle. *J. Appl. Physiol.* 90:1119–1124.

Pette D (2002). The adaptive potential of skeletal muscle fibers. *Can. J. Appl. Physiol.* 27:423–448.

Pette D, Staron RS (1997). Mammalian skeletal muscle fibre type transitions. *Int Rev Cytol* 170:143–223.

Pette D, Staron RS (2001). Transitions of muscle fibre phenotypic profiles. *Histochem. Cell Biol.* 115:359–372.

Reader M, Schwartz G, English AW (2001). Brief exposure to testosterone is sufficient to induce sex differences in the rabbit masseter muscle. *Cells Tissues Organs* 169:210–217.

Roy RR, Baldwin KM, Edgerton VR (1991). The plasticity of skeletal muscle: Effects of neuromuscular activity. *Exerc. Sport Sci. Rev.* 19:269–312.

Sant'ana Pereira JA, Wessels A, Nijtmans L, Moorman AFM, Sargeant AJ (1995). New method for the accurate characterization of single human skeletal muscle fibres demonstrates a relation between mATPase and MyHC expression in pure and hybrid fibre types. *J. Muscle Res. Cell Motil* 16:21–34.

Schiaffino S, Reggiani C (1994). Myosin isoforms in mammalian skeletal muscle. *J. Appl. Physiol.* 77:493–501.

Sciote JJ, Rowlerson AM, Hopper C, Hunt NP (1994). Fibre type classification and myosin isoforms in the human masseter muscle. *J. Neurol. Sci.* 126:15–24.

Sciote JJ, Kentish JC (1996). Unloaded shortening velocities of rabbit masseter muscle fibres expressing skeletal or alpha-cardiac myosin heavy chains. *J. Physiol.* 492:659–667.

Sciote JJ, Horton MJ, Rowlerson AM, Link J (2003). Specialized cranial muscles: how different are they from limb and abdominal muscles? *Cells Tissues Organs* 174:73–86.

Scutter SD, Türker KS (1998). Recruitment stability in masseter motor units during isometric voluntary contractions. *Muscle Nerve* 21:1290–1298.

Sfondrini G, Reggiani C, Gandini P, Bovenzi R, Pellegrino MA (1996). Adaptations of masticatory muscles to a hyperpropulsive appliance in the rat. *Am. J. Orthod. Dentofacial Orthop.* 110:612–617.

Staron RS (1991). Correlation between myofibrillar ATPase activity and myosin heavy chain composition in single human muscle fibers. *Histochemistry* 96:21–24.

Staron RS, Karapondo DL, Kraemer WJ, Fry AC, Gordon SE, Falkel JE, Hagerman FC, Hikida RS (1994). Skeletal muscle adaptations during early phase of heavy-resistance training in men and women. *J. Appl. Physiol.* 76:1247–1255.

Stephenson GMM (2001). Hybrid skeletal muscle fibres: a rare or common phenomenon? *Clin. Exp. Pharmacol. Physiol.* 28:692–702.

Thomason DB, Booth FW (1990). Atrophy of the soleus muscle by hindlimb unweighting. *J. Appl. Physiol.* 68:1–12.

Tipton KD, Ferrando AA, Williams BD, Wolfe RR (1996). Muscle protein metabolism in female swimmers after a combination of resistance and endurance exercise. *J. Appl. Physiol.* 81:2034–2038.

van Wessel T, Langenbach GEJ, Korfage JAM, Brugman P, Kawai N, Tanaka E, van Eijden TMGJ (2005). Fibre-type composition of rabbit jaw muscles is related to their daily activity. *Eur. J. Neurosci.* 22:2783–2791.

Weijs WA, Brugman P, Grimbergen CA (1989). Jaw movements and muscle activity during mastication in growing rabbits. *Anat. Rec.* 224:407–416.

Widmer CG, Carrasco DI, English AW (2003). Differential activation of neuromuscular compartments in the rabbit masseter muscle during different oral behaviors. *Exp. Brain Res.* 150:297–307.

Widrick JJ, Trappe SW, Costill DL, Fitts RH (1996). Force-velocity and force-power properties of single muscle fibers from elite master runners and sedentary men. *Am. J. Physiol.* 271:C676–C683.

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter IV

The Use of Rabbits to Investigate the Pathogenesis of Disease

C. S. Thompson*
Department of Surgery, Division of Surgery and Interventional Science,
University College London Medical School,
Royal Free Campus, London

Abstract

The rabbit (Oryctolagus cuniculus) belongs to the taxonomic order Lagomorpha and exists in three forms: wild, feral and domestic. This good natured and medium-sized animal is widely used in biomedicine to investigate many aspects of human disease. Data generated from rabbits have provided researchers with a wealth of useful scientific information. Pharmacological, surgical, immune and genetic investigations have reinforced the notion that they have physiological and anatomical similarities to man, facts that can be exploited in disease prevention and treatment studies.

In recent years the scope of their use has increased dramatically and now covers a myriad of human conditions: cerebrovascular disease including Alzheimer's, cardiovascular, reproductive including erectile dysfunction, lung, liver, kidney and bone diseases, as well as viral infection.

Development of transgenic rabbit models has also opened up new opportunities to look at disease-induced alterations in the genome. This chapter describes these models and evaluates their usefulness in reproducing the clinical characteristics and treatment of various human diseases.

Introduction

The use of animal models in biomedical science, whilst controversial remains a very powerful tool in understanding various human diseases, in developing new medical/surgical

* Tel.+44 207 7940500 x 33440. E.mail address: cecil.thompson@nhs.net; cecil.thompson@ucl.ac.uk.

procedures and in evaluating therapeutic interventions. Animal models also have the advantage that they eliminate factors such as ethnicity, economic and geographic variables, drug interaction, diet, gender and age differences that limit clinical studies [1] In this regard, the rabbit model has found prominence due to physiological and anatomical similarities to humans, making it suitable for the study of different human pathologies [2]. Although rabbits appear rodent-like in some morphological features, protein sequencing data suggests that they may be more closely related to primates than rodents [3], which may account for the perceived similarities to humans.

The ancestral home of the European rabbit (Oryctolagus cuniculus, part of the taxonomic order Lagomorpha), is believed to be the Iberian Peninsula [4]. There are around 35 breeds and more than double that number of varieties in Great Britain, which exist in three forms: wild, feral and domestic [4].

The New Zealand White rabbit is the most commonly used in scientific studies and to a lesser extent the Dutch Belted and the Flemish Giant breeds. The breeds also show variation in their body weight, ranging from about 1kg for the Netherland Dwarf, to 6 kg or more for the Flemish Giant [4]. They are not generally kept longer than 4 years under laboratory and commercial conditions, as their physiological functions deteriorates with age [4]. Another factor that encourages their use in scientific studies is derived from the wealth of biological information in the literature on rabbit blood, urine, cerebrospinal, synovial, digestive and reproductive secretions [4], which can be compared to disease-induced changes.

Traditionally, rabbits have been used in the production of polyclonal and monoclonal antibodies [2], surgical procedures [5] and teratogenicity testing of pharmaceutical compounds [6]. However, in recent years the scope of their use has increased dramatically and now covers a myriad of human conditions: cerebrovascular disease including Alzheimer's, cardiovascular, reproductive including erectile dysfunction, lung, liver, kidney and bone diseases, as well as viral infection.

Development of transgenic rabbit models has also opened up new opportunities to look at disease-induced alterations in the genome. This chapter describes these models and evaluates their usefulness in reproducing the clinical characteristics of several diseases, which may help in the formation of new treatment strategies.

1. Cerebrovascular Disease

Cerebrovascular disease is a group of brain dysfunctions related to disease of blood vessels supplying the brain. These include stroke, which is a focal neurologic deficit caused by a change in cerebral circulation; the second leading cause of death worldwide [7]. It results from ischemic (brain infarction), as well as, intracerebral or subarachnoidal hemorrhage [8]. Approximately 80% of all strokes are ischemic, 10% are due to intracerebral hemorrhage, and the rest are subarachnoidal hemorrhages [8].

The magnitude of the stroke burden on the healthcare system necessitates urgent work towards developing novel therapies, including neuroprotective drugs, safer and more effective thrombolytic drugs, mechanical interventions, and restorative (neuroregenerative) agents.

1.1. Ischemic Stroke

Ischemic stroke is often caused by occlusion of the middle cerebral artery or one of its branches. This can be mimicked in rabbits, resulting in permanent ischemia in a controlled manner [9]. The occlusion limits the blood capacity to deliver oxygen causing cerebral tissue damage.

A potential treatment option that has been investigated in rabbits involves the intravenous administration of dodecafluoropentane emulsion, an efficient transporter of oxygen. This agent decreases infarct volume by protecting the rabbit brain tissue from ischemia possibly by decreasing the degree of hypoxia [9]. Thromboembolic stroke, the most common in humans, has also been mimicked in rabbits to test thrombolytic agents [10-12], as well as to investigate the consequences of thrombolysis, such as hemorrhagic transformation [13].

1.2. Hemorrhagic Stroke

Strokes caused by intracerebral and subarachnoid hemorrhages are associated with high mortality and most survivors are burdened with severe disability. Rabbits have been used to investigate intracerebral hemorrhages [14], the most common model involves autologous blood injection into the desired brain region [15, 16]. Similarly, subarachnoid hemorrhages have also been induced in the rabbit [17-20]. Oxidative stress and brain edema are thought to be contributing factors in the development of the subsequent brain injury. This is supported by the finding that antioxidants, such as alpha lipoic acid and saline saturated with hydrogen, attenuate this type of brain injury by reducing oxidative stress [18, 19].

1.3. Alzheimer’s Disease

Alzheimer’s disease is a neurodegenerative, late-onset disorder of the brain, characterized clinically by a progressive disturbance in memory, judgement, reasoning and olfaction [21]. Pathologically, this results in changes in brain structure, including increased ventricular volume, extracellular accumulation of amyloid beta protein containing plaques, and loss of synaptic connectivity/integrity [21, 22]. Although, the vast majority of cases are sporadic with unknown etiology, altered cholesterol homeostasis and oxidative stress are now considered risk factors [21-23].

Autopsy studies; have also highlighted a possible link between heart disease, cholesterol and Alzheimer’s disease [24, 25]. Many of the markers associated with Alzheimer’s disease have been observed in cholesterol-fed rabbits [22], including central accumulation of amyloid beta and cognitive deficits [24, 25], as well as increased oxidative stress [23]. Indeed, removal of dietary cholesterol or treating the rabbit with cholesterol-lowering drugs [25, 26], as well as reducing oxidative stress with caffeine [23] have all been shown to reverse/reduce the severity of Alzheimer’s disease.

In general, rabbit models of stroke and Alzheimer’s disease are able to reproduce important pathophysiological events relevant to the human condition, which potentially can help in the development of novel treatment regimes.

2. Cardiovascular Disease

Approximately one-third of the total deaths worldwide per annum, amounting to over 17 million people, are due to cardiovascular disease [27], making it the leading cause of death [28], with a major impact on morbidity [27]. Complications arising from this disease are due to complex multifactorial pathologies, in which genetic and environmental factors are implicated. Rabbit models have been developed to replicate many of the features of human cardiovascular disease.

2.1. Atherosclerosis

Atherosclerosis is a chronic inflammatory disorder and the underlying risk factor for most cardiovascular disease [29]. The rabbit has become an important animal model to study human development of the disease [30]. Cholesterol-fed rabbits are popular, since they accumulate large amounts of cholesterol in their plasma [30] and develop atherosclerotic changes in the arterial intima, similar to humans [31]. The Watanabe heritable hyperlipidemic (WHHL) rabbit develops hypercholesterolemia due to a binding-defective mutation of the low density lipoprotein receptor, with atherosclerotic progression similar to human familial hypercholesterolemia [32]. These animals have been used to study destabilization of atheromatous plaques and to evaluate drugs that reduce plasma cholesterol levels and suppress atherosclerosis. Spontaneous myocardial infarction-prone WHHL rabbits are another strain developed by selective breeding of coronary atherosclerosis-prone WHHL rabbits. These animals have severely stenosed coronary arteries due to atherosclerotic lesions, similar to patients with coronary heart disease [33].

Dyslipidemia treatment regimes using cholesterol-fed rabbits have primarily focussed on reducing hypercholesterolemia with drugs such as HMG-CoA reductase inhibitors (statins). Atorvastatin prevented atherosclerotic changes in rabbits fed high cholesterol diets by lowering plasma lipids [34], as well as by cholesterol-independent mechanisms [35] and by an antioxidant action [36]. Fluvastatin in combination with the AT1 receptor antagonist losartan also inhibited atherosclerotic development and reduced inflammation in a similar rabbit model [37]. In addition, paditaxel-eluting stents surgically placed in the thoracic aorta of cholesterol-fed rabbits simultaneously given atorvastatin, resulted in a marked improvement of advanced atherosclerosis [38]. Ezetimibe, a potent inhibitor of sterol absorption that selectively blocks the uptake of dietary cholesterol in the small intestine also inhibited atherosclerosis [39]. Similarly, WHHL rabbits given the antioxidant probucol [40], the AT1 receptor blocker irbesartan [41] or the neurohypophyseal peptide oxytocin, which inhibits inflammation [42] all showed reduced progression of atherosclerosis.

2.2. Diabetes Mellitus

Diabetes mellitus is a chronic condition where blood glucose concentration is uncontrollably high, due to impaired insulin homeostasis. Type 1 diabetes mellitus (T1DM or insulin dependent diabetes mellitus) is caused by a reduction in circulating insulin [43, 44].

Type 2 diabetes mellitus (T2DM or non-insulin dependent diabetes mellitus) results from insulin resistance, so that even if the absolute insulin concentration is normal or elevated, it is inappropriately low for the prevailing circulating glucose concentration [45,46]. The global prevalence of diabetes mellitus in 2000 was estimated to be 171 million, a figure predicted to double by 2030, due to population growth, aging, urbanisation, increasing obesity and physical inactivity [47].

The New Zealand White rabbit is known to develop diabetes mellitus spontaneously [48], however, most studies use experimental diabetic models, where the pancreatic β-cells have been selectively destroyed with the cytotoxic chemical agent, alloxan [49,50]. The diabetic condition was significantly ameliorated when these rabbits were given glibenclamide, the most potent sulfonylurea [51]. While, orally administed nanoparticles containing glibenclamide produced a better release profile, with enhanced efficacy and bioavailability compared to conventional glibenclamide [52]. Similarly, plant extracts from caralluma sinaica and catharanthus roseus (Apocyanaceae) were as effective as glibenclamide in reducing blood sugar levels in diabetic rabbits [53,54]. The antidiabetic action of metformin, another popular antidiabetic drug has also been demonstrated in this model [55].

2.3. Cardiac Disease

Cardiac disease such as myocardial infarction and dilated cardiomyopathy contribute to cardiovascular-induced morbidity and mortality.

Myocardial infarction is caused by a blockage in coronary arteries, primarily due to atherosclerosis or thrombosis [56] and is characterized by necrosis of myocardiocytes due to a reduction in cardiac blood supply. Conventional treatment such as percutaneous coronary intervention, coronary-artery bypass graft surgery and anti- or dissolution- thrombotic therapy can reduce death rate to a certain extent [57].

Myocardial infarction leading to acute ischemia has been created in rabbits using several techniques. One method involves the partial ligation of the descending coronary artery [58, 59]. While other methods include endovascular coil occlusion of the marginal artery [60], balloon occlusion developed from percutanous translaminal coronary angioplasty [61], and the percutaneous, minimally-invasive, closed-chest model [62]. The antihypertensive drug ramipril, an angiotensin-converting enzyme inhibitor, decreased the incidence of myocardial infarction-induced ventricular tachycardia and fibrillation in rabbits [63]. The atherosclerosis-prone WHHL rabbit strain can also be used to studying many aspects of coronary heart disease [33].

Stem cell therapy has emerged as a promising treatment for cardiac and vascular repair. This form of treatment aims to rebuild functional myocardium by transplanting exogenous stem cells or by activating native stem cells to induce endogenous repair [64]. Granulocyte colony stimulating factor improved the survival of rabbits with myocardial infarction by inducing bone marrow stem cell mobilization and homing to infarcted areas [65].

Dilated cardiomyopathy is a primary myocardial disease characterized by chamber dilation associated with impaired systolic and diastolic function [66], which can lead to congestive heart failure. Matsui et al. described a rabbit model of dilated cardiomyopathy producing morphological changes in the heart similar to those found in the human disease by immunization with synthetic peptides [67]. Doxorubicin used as a chemotherapeutic agent,

has also been reported to induce cardiotoxicity causing dilated cardiomyopathy in rabbits [68]. Rabbit models will continue to play an important role in cardio-pathophysiology research, especially as stem cell research moves forward.

3. Lung Disease

Asthma, chronic obstructive lung disease, chronic bronchitis and emphysema are primary examples of the pathological consequences of chronic lung disease and are usually associated with airway inflammation, airway hyperresponsiveness and mucous overproduction [69]. The similarity between rabbits and humans in terms of airway anatomy and responses to inflammatory mediators exemplifies the value of this species in the investigation of the pathophysiology of lung disease.

3.1. Asthma

Asthma is due to reversible airway obstruction, airway inflammation, and increased airway responsiveness to both physical and chemical stimuli [70]. Many of these features are found in rabbit asthmatic models, which are often sensitive to the same drugs as humans [69]. Endogenous glucocorticoids suppress the asthmatic state induced in rabbits following inhalation of the proasthmatic and proinflammatory cytokine, IL-13 [71]. G proteins, in particular the Gβγ subunit of Gi protein, regulate the allergic asthmatic phenotype, including the induction of airway hyperresponsiveness and inflammation. This response was suppressed by pretreatment with an inhaled membrane-permeable anti-Gβγ blocking peptide (similar to glucocorticoid pre-treatment) in the ovalbumin sensitized rabbit, a model of allergic asthma [72]. The inflammatory response seen in asthmatic patients include infiltration of inflammatory cells into lung tissues, high levels of IgE and IgG1 in sera, thickening of airway epithelial tissue, and excessive mucous secretion due to goblet cell hyperplasia and hypertrophy of mucous glands [69].

3.2. Lung Inflammation

Lung inflammation can be induced in rabbits by direct intratracheal instillation of particulate matter. The antiinflammatory effect of lovastatin attenuated the recruitment and activation of alveolar macrophages and polymorphonuclear leukocytes, reducing local proinflammatory cytokine production, and promoting the clearance of particulate matter from lung tissues [73]. Anti-inflammatory and antioxidant treatment with glucocorticoids and N-acetylcysteine, respectively, reduced pulmonary surfactant dysfunction in the rabbit meconium-induced oxidative lung injury model [74]. Moreover, the selective PDE3 inhibitor olprinone, demonstrated potent antioxidative and anti-inflammatory properties in the same rabbit model [75]. Further evidence that oxidative stress plays an important role in the development of lung injury comes from the finding that tetomilast, a potent inhibitor of

superoxide production and oxidative stress, protected rabbits against porcine pancreatic elastase-induced emphysema [76].

3.3. Acute Lung Injury and Acute Respiratory Distress Syndrome

Acute lung injury and acute respiratory distress syndrome are clinical syndromes defined by varying degrees of acute hypoxemic respiratory failure, pulmonary infiltrates, pulmonary edema (influx of protein-rich fluid into the alveolar spaces) and poor lung compliance. Conventional mechanical ventilation therapy is fundamental in acute respiratory distress syndrome treatment. Inhaled nitric oxide has been used clinically to improve oxygenation and reduce lung injury in these syndromes. Acute lung injury and oxidative stress can be induced in rabbits by tracheal infusion of warm saline. Inhaled nitric oxide attenuated oxidative stress and histopathological and inflammatory lung injury in these animals [77].

3.4. Stem Cell Therapy

It is now recognised that the rabbit serves as a good platform to evaluate stem-cell-based therapies [69]. The purpose of stem cell transplantation is to replace damaged or lost cells in an organ or tissue and genetic correction.

Most organs contain their own small reservoir of stem cells, also called progenitor cells, which are recruited to start dividing and replace cells that have died during normal aging or to repair small areas of damage. Several potential sources of progenitor cells for airway epithelium have been identified, including exogenous stem cells [78]. The ability to repair tissue by endogenous lung epithelial progenitor cells is often insufficient as the natural repair capacity appears to diminish with age [79]. Autologous transplantation of peripheral blood-derived circulating endothelial progenitor cells partly restored pulmonary endothelial function and effectively attenuated endotoxin-induced acute lung injury in rabbits by direct endothelial repair and indirect immunomodulation of antioxidation and antiinflammation (80).

4. Liver Disease

4.1. Nonalchoholic Fatty Liver Disease

Nonalchoholic fatty liver disease covers a spectrum of disorders from simple steatosis to nonalcoholic steatohepatitis, which develop without excessive alcohol consumption [81,82]. Increasingly, it is being recognized as a problem not only in developed countries but worldwide [83]. The hallmark of the disease is accumulation of intracytoplasmic triglyceride within hepatocytes that can ultimately lead to cirrhosis and liver failure, as well as hepatocellular carcinoma [81,82].

Ogawa et al. created a model of steatohepatitis, in which advanced fibrosis (pre-cirrhosis stage) was produced by feeding rabbits a high fat diet [82]. They noted that ezetimibe, which blocks intestinal cholesterol absorption from the diet attenuated steatohepatitis, suggesting its

clinical use for treating human liver diseases caused by elevated cholesterol [82]. The intragastric administration of carbon tetrachloride induced chronic liver damage, similar to primary hepatic cirrhosis [84,85]. In this model, collagenase supplementation via portal vein infusion retarded hepatic cirrhosis development and caused the regression of established cirrhosis [86].

4.2. Acute Hepatic Failure

Acute hepatic failure is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with high mortality. Popular methods for creating this disease in rabbits include the use of hepatotoxic chemical agents such as acetaminophen (paracetamol) and galactosamine, as well as infective procedures, which reproduce a number of important clinical characteristics of acute hepatic failure [84].

D-galactosamine can cause serious metabolic alterations and hepatic necrosis, including histological and biochemical changes in the rabbit compatible with acute hepatic failure [87]. Allogeneic mononuclear bone marrow cell transplantation at multiple sites in the liver was found to reduce galactosamine-induced liver pathology, suggesting that this therapy is an effective way to repair liver injury [88].

Acute hepatic failure can also occur by infecting rabbits with rabbit hemorrhagic disease virus [89]. The viral antigen was found in hepatocytes post-infection [90] and the rabbits had clinical signs characteristic of human acute hepatic failure [89]. N-acetylcysteine and melatonin were both found to be hepatoprotective in this model, due to modulation of apoptosis [91, 92].

4.3. Hepatic Ischemia-Reperfusion Injury

The morbidity associated with liver transplantation and major hepatic resections is partly a result of ischemia-reperfusion injury [93]. Reperfusion of a previously ischemic organ triggers a cascade, collectively termed ischemia–reperfusion injury that leads to production of reactive oxygen species and an increased secretion of systemic inflammatory mediators [94,95]. Hepatic ischemia-reperfusion injury can be created in rabbits by liver lobar ischemia followed by reperfusion [96,97].

Propofol a short-acting, hypnotic/amnestic agent known to improve hepatic perfusion in the rabbit decreased the leakage of liver enzymes and markedly reduced histological lesions following hepatic injury [96]. Similarly, the non-essential amino acid glycine reduced the injury by reducing the systemic inflammatory response, and maintaining cellular energy production [97].

The knowledge gained from animal models of hepatic ischemia-reperfusion injury has the potential to improve the outcome of patients following hepatic surgery.

5. Kidney Disease

Acute Kidney Injury and Chronic Kidney Disease

Patients with acute kidney injury may require renal replacement therapy and have an increased risk of chronic kidney disease [98]. Glomerular hyperfiltration, hypertension and proteinuria are key mediators of chronic kidney disease and are associated with activation of proinflammatory and profibrotic signaling pathways in proximal tubular epithelial cells, which can progress to end-stage kidney disease [99].

Both acute kidney injury and chronic kidney disease are now recognized as integrated disorders that share common pathways, such as cell death, proliferation and differentiation, inflammation and fibrosis. Acute kidney injury is caused by many events including; ischemic episodes bacterial toxins and urinary tract obstruction [98], while hypertension and diabetic nephropathy are the main causes of chronic kidney disease in adults [100].

Although, very little information has been generated on rabbit models of chronic kidney disease, Lau et al. found diabetic nephropathy developed in alloxan-treated rabbits characterized by elevated serum creatinine and proteinuria, as well as a decrease in creatinine clearance [101]. These variables were normalized with vardenafil, a phosphodiesterase type 5 inhibitor commonly used to treat erectile dysfunction. Rabbit studies on acute renal injury have mainly focussed on the renal ischemia-reperfusion model. Yakut et al found that iloprost, a stable prostacyclin analog, inhibited oxygen-free radical production and levosimendan, which has anti-inflammatory and anti-apoptotic properties, were both effective treatment options for acute renal injury [102]. Further support for the role of reactive oxygen metabolites in this disease comes from the finding that Renshen polysaccharides increased endogenous antioxidants catalase and superoxide dismutase, decreasing renal oxidative injury in this rabbit model [103].

Bacterial infection caused by the enterohemorrhagic strain of Escherichia coli is known to cause renal disease in humans. This can also be investigated in the rabbit, since Dutch Belted rabbits infected with this bacterium develop hemolytic-uremic syndrome-like disease [104].

6. Reproductive Disease and Erectile Dysfunction

6.1. Endometriosis

Endometriosis is a common, hormone-dependent gynecological disease, resulting in growth of endometrial glands and stroma outside the uterus. It is an inflammatory condition associated with elevated reactive oxygen species, infertility and pain [105]. Systemic administration of progesterone has been used to simulate pregnancy, relieving pain and control disease progression, however, this form of treatment has poor compliance because of side effects [106].

Yuan et al. [106] created endometriosis in rabbits and found that those treated with an intracystic injection of levonorgestrel-loaded polylactic acid microspheres achieved high levels of drug localized in the endometrial cysts: these caused a reduction in size as well as

endometrial atrophy of the cysts. This study suggests that this form of treatment may be an alternative to surgery.

6.2. Ectopic Pregnancy

An ectopic pregnancy is where the fetus develops outside the uterus, typically in the fallopian tube and is the leading cause of maternal deaths during early pregnancy [107]. Suitable animal models to investigate this disorder are rare, as this is essentially a human disorder; nevertheless, rabbits at mid pregnancy were used to investigate the feasibility of intrachorionic injection therapy or hyperthermia in ectopic pregnancy. The application of hyperthermia or intrachorionic injection therapy terminated the ectopic products of gestations, suggesting this mode of treatment has clinical potential [108].

6.3. Preterm Birth

In humans, preterm birth refers to a birth prior to the 37th week of gestation and occurs in more than 10% of all pregnancies. It is considered a global risk factor threatening women's health, with 60%–80% perinatal mortality, and is frequently associated with preeclampsia (characterized by hypertension and proteinuria), infections and fetal abnormalities [109].

Gorenberg et al., found that pregnant rabbits injected with RU486 (mifepristone; a synthetic steroid progesterone receptor antagonist used as an abortifacient) caused non-infection-mediated preterm birth [110]. Pregnant rabbits inoculated transcervically with prevotella bivia associated with bacterial vaginosis, developed a chronic intrauterine and fetal infection that was accompanied by preterm birth in up to 33% of cases [111]. Premature birth in pregnant rabbits also occurred following transcervical inoculation of Escherichia coli. Antibiotic treatment with ampicillin-sulbactam (a combination of the common penicillin-derived antibiotic and an inhibitor of bacterial beta-lactamase, respectively) given to these rabbits prolonged pregnancy and reduced perinatal mortality if administered early [112]. Similar results were obtained following treatment with recombinant human lactoferrin, which has an anti-inflammatory action, as well as an antibacterial action [113].

6.4. Feto-Placental Development

Embryonic and feto-placental development in the rabbit is similar to humans, making it a particularly suitable model to investigate the importance of embryo and fetal development on offspring and adult health. Insights into human reproduction have been obtained from the rabbit, due to exact staging of early embryonic developmental and maternal pregnancy and the similarity of placental morphology and function between both species [114].

The growth of the fetus is determined by maternal diet and hypercholesterolemia can cause increased fatty streak formation in human fetal arteries and accelerate atherosclerosis during childhood [115]. This is also the case in rabbits; maternal hypercholesterolemia increased postnatal atherogenesis, whereas lowering maternal plasma cholesterol and antioxidant treatment greatly reduced fetal and postnatal atherogenesis [115]. It is likely that

placental permeability to lipoproteins is affected, since increased amounts of collagen were observed in fetal tissues when the maternal rabbits had hypercholesterolemia. This alteration results in increased susceptibility to atherosclerosis in adult life, representing a risk factor for the early development of disease, which may appear even in the prenatal period (116). These findings stress the importance of prenatal control of lipoprotein levels, as a preventative factor against the early development of atherosclerosis.

Selective ligature of pregnant rabbit uteroplacental vessels reproduced similar cardiovascular features to those observed in human fetuses with intrauterine growth restriction [117]; this led to increased mortality and histological brain changes [118]. It has also been observed that rabbits with mild and moderate maternal hypertension differentially alter placental structure and gene expression, affecting placental functional capacity, which may contribute to the programming of hypertension in the offspring [119].

6.5. Erectile Dysfunction

Penile erection is a hemodynamic process involving increased arterial inflow and restricted venous outflow, co-ordinated with corpus cavernosum smooth muscle relaxation. [120]. NO is an important mediator of this process, as it initiates corpus cavernosal smooth muscle relaxation through the formation of cGMP [121,122]. Erectile dysfunction is a condition where impaired corpus cavernosal relaxation has developed due to a reduction in the cGMP/NO pathway. It is defined as the persistent inability to attain and maintain an erection adequate to permit satisfactory sexual performance and can affect up to 50% of men aged between 40 –70 years old [123]. The association between erectile dysfunction and cardiovascular disease has confirmed that it is another manifestation of atherosclerotic vascular disease with the same risk factors, such as hypertension, dyslipidemia, diabetes mellitus and smoking [120,124].

The structure of the rabbit penis is similar to humans and several rabbit models have been developed to investigate the physiological and biochemical nature of erectile dysfunction [125]. The anesthetized rabbit is useful, since it allows the administration of drugs by several routes, including intracavernous injection, as well as hemodynamic recordings in parallel with intracavernosal pressure measurements, an indicator of penile erection [125].

Stimulation of the cavernous nerve caused an erection in these rabbits [126] as did intracavernosal injection of sildenafil [127], while animals with vasculogenic impotence showed evidence of intracavernosal pressure decay [128].

The conscious rabbit model can also be used to assess compounds with potential for treating erectile dysfunction, in particular the efficacy and mechanism of PDE5 inhibitors [129,130]. The PDE5 inhibitor DA-8159 enhanced the erectile activity of conscious rabbits following surgical transection of the spinal cord, highlighting the potential for drug-induced preservation of erectile function in patients with spinal cord injury [131].

The prevalence of erectile dysfunction in diabetic men is up to 70%, highlighting the magnitude of the problem in this patient group [132, 133]. Studies using diabetic rabbits confirm that this is due to a reduction in cGMP/NO levels and corpus cavernosal smooth muscle relaxation [134]. The accumulation of cGMP following treatment with PDE5 inhibitors and the subsequent increase in smooth muscle relaxation is crucial in treating diabetic erectile dysfunction [134, 135].

It is recognised that benign prostatic hyperplasia can cause partial bladder outlet obstruction [136], linked to erectile dysfunction [137,138], where sexual performance is related to the severity of the prostatic hyperplasia [139]. The clinical consequence of these associations can be reproduced in rabbits by tying a ligature around the proximal urethra at the base of the bladder neck [140]. This resulted in erectile dysfunction [141,142], which was improved by losartan and vardenafil [142].

7. Bone Disease

7.1. Osteoporosis

Osteoporosis the most common bone disease develops because of low bone mineral density and deterioration of bone structure, as a result the bone becomes weak and fractures easily, especially in postmenopausal women [143]. It can be reproduced in rabbits following bilateral ovariectomy with methylprednisolone treatment [144], or by giving methylprednisolone [145] or dexamethasone [146] alone. Dental implant osseointegration can be impaired in medical conditions where bone mass is reduced, such as glucocorticoid-induced osteoporosis. This condition was alleviated by intermittent parathyroid hormone treatment, which enhanced the bone response around titanium dental implants placed in the proximal tibia of female rabbits [144]; similar findings were observed following treatment with the bisphosphonate zoledronic acid [145] and ibandronate [146].

7.2. Osteomyelitis

Osteomyelitis is an acute or chronic inflammation of the bone secondary to a bacterial or fungal infection, which can be a complication of injury or surgery.

Tibial osteomyelitis has been induced in rabbits using methicillin-resistive staphylococcus aureus bacterial infection. Leukocyte- and platelet-rich plasma gel, used to enhance tissue healing was found to exhibit antimicrobial efficacy in this model, suggesting that a combination of the plasma gel and antibiotics could be a favorable mode of treating osteomyelitis [147]. Bacterial-induced osteomyelitis was also treated in rabbits using polymethylmethacrylate, a matrix-based antibiotic delivery system with xylitol. The xylitol increased the porosity of polymethylmethacrylate, which enhanced the antibiotic elution [148].

7.3. Osteonecrosis

Osteonecrosis is a condition that develops as a consequence of temporary or permanent loss of blood supply to bone, which results in that part of the bone dying by necrosis and apoptosis. Rabbits with steroid-induced osteonecrosis were successfully treated with granulocyte colony-stimulating factor and stem cell factor [149]. While transplantation of hepatocyte growth factor-transgenic mesenchymal stem cells promoted recovery from

osteonecrosis of the femoral head in rabbits [150]. Equally, cryopreserved autologous bone marrow-derived mononuclear cells, which contain bone marrow-derived stem cells, also promoted bone regeneration and neovascularization in these rabbits [151].

8. Viral Infection

Viruses can be spread by simple contact, exchanges of saliva, coughing, or sneezing. Some require sexual contact, while others go through the fecal-oral route via contaminated food or water, or require an insect to carry them from person to person. Treatments for viral infections require either vaccination for prevention or antiviral drugs, which inhibit their progression. The rabbit has been used to investigate several viral infections. The threat of bioterrorism has caused concern that smallpox infection may be reintroduced. Only rabbitpox virus infection in rabbits shows patterns of natural airborne transmission similar to smallpox [152]. Using this model, the antiviral drug ST-246 or the vaccine Imvamune were found to be highly effective in protecting rabbits against the infection [153, 154]. The antiviral drug CMX001, also afforded effective protection against rabbitpox, supporting the notion that this form of treatment could be feasibility for orthopox virus infections in humans [155]. Monoclonal antibodies against smallpox are also effective against rabbitpox, which may control smallpox disease in immunocompetent or immunodeficient humans [156].

Hepatitis E virus is a major cause of enterically transmitted human hepatitis; the rabbit is also susceptible to this viral infection but can be protected by HEV p179 vaccine action [157]. Herpes simplex virus type 1 epithelial keratitis is another viral infection that can be transmitted to rabbits. Geldanamycin the antibiotic with broad-spectrum antiviral action significantly reduced the severity of this infection [158]. Finally, topical dexamethasone minimized the clinical symptoms of adenovirus infection in rabbit eyes, a potential treatment for epidemic adenoviral keratoconjunctivitis [159].

9. Transgenic Rabbits

The development of novel transgenic tools adapted to the laboratory rabbit has opened new opportunities in precise tissue and developmental stage specific gene expression [2]. Transgenic rabbits are now a well used model for several diseases, since by introducing genetic mutations of human genes, rabbit models can more accurately reflect human pathophysiology [160-162]. To date, cardiovascular diseases have been most studied, the transgenic rabbit models providing insight into atherogenesis and lipoprotein metabolism. About a dozen proteins have been expressed, helping to elucidate their metabolic role in atherosclerosis [162]. They include apolipoproteins affecting low-density and high-density lipoprotein concentrations and enzymes (hepatic lipase, lipoprotein lipase and lecithin-cholesterol acyltranferase), which alter the metabolism and plasma profiles of the different lipoprotein classes [162].

Elevated levels of plasma high-density lipoproteins are associated with a low incidence of cardiovascular disease. These lipoproteins contain apolipoprotein AI and AII. It is generally accepted that apolipoprotein AI provides protection against atherosclerosis. However, the role

of apolipoprotein AII is unclear; although transgenic rabbits expressing human apolipoprotein AII develop combined hyperlipidemia and have markedly reduced plasma high-density lipoproteins [163], there is still retention of atheroprotective properties [164]. Apolipoprotein CIII has been implicated in hypertriglyceridemia and is an independent risk factor for coronary heart disease. Transgenic rabbits overexpressing the human apolipoprotein CIII gene develop hypertriglyceridemia, similar to that in patients [165]. Although, these studies provide evidence that the transgenic rabbit is a valuable tool to study human hyperlipidemia in relation to cardiovascular disease, more work is required to elucidate their exact role.

Long QT syndrome is a heritable disease associated with electrocardiograph QT interval prolongation, ventricular tachycardia, and sudden cardiac death in young patients [166]. Transgenic rabbits expressing the human genes for Long QT have been used as a model to test potential side effects of anesthetic agents in subjects genetically predisposed to sudden cardiac death [167] and evaluate therapeutic compounds for the treatment or prevention of Long QT syndrome [168,169].

The mammalian heart expresses 2 cardiac myosin isoforms, α- and β- myosin heavy chain. A decrease in the α-myosin heavy chain is a common feature of human heart failure, while overexpression in transgenic rabbits proved to be cardioprotective against tachycardia-induced cardiomyopathy [170].

Retinitis pigmentosa is the name given to a group of inherited retinal disorders characterized by a progressive loss of rod and cone photoreceptors and eventual atrophy of the entire retina. A transgenic rabbit model of retinal degeneration has been generated by pronuclear injection of the bacterial artificial chromosome transgenic construct into their embryos [171]. Transcorneal electrical stimulation of photoreceptors was found to be neuroprotective, as it slowed the rate of decrease of the electroretinogram in these rabbits [172].

Conclusion

Rabbit models have, and continue to play, a pivotal role in understanding the pathophysiological consequences of human disease progression and developing treatment strategies. These models mimic many of the features of human disease and as such are appropriate and effective research tools. However, as no animal model fully represents all aspects of human disease the challenge for future researchers will be to develop new rabbit models to further dissect the complexities and pathomechanisms of disease. Each model should be carefully selected in accordance with the clinical problem under investigation, while the therapeutic recommendations derived from such studies should be restricted to each particular pathophysiological situation.

The use of transgenic rabbits is relatively new and has not been fully exploited. However, it has the potential to open up areas of genome research, which may revolutionize genetics. It is likely that translating laboratory work using rabbits into clinical practice will lead to major breakthroughs in the treatment of many diseases in the future.

References

[1] Potenza MA, Nacci C, Gagliardi S, Montagnani M. Cardiovascular complications in diabetes: lessons from animal models. *Curr. Med Chem* 2011; 18: 1806-1819.

[2] Duranthon V, Beaujean N, Brunner M, Odening KE, Navarrete Santos A, Kacskovics I, Hiripi L, Weinstein EJ, Bosze Z. On the emerging role of rabbits as human disease model and the instrumental role of novel transgenic tools. *Transgenic Res* 2012; 21: 699-713.

[3] Graur D, Duret L, Gouy M. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). *Nature* 1996; 379: 333-335.

[4] Adams CE. The laboratory Rabbit. Chapter 26, In The UFAW Handbook on "The care & management of laboratory animals". Ed. Trevor Poole, Sixth edition, Longman *Scientific & Technical* 1987, pp 415-435.

[5] Ogawa T, Ishii T, Mishima H, Sakai S, Watanabe A, Nishino T, Ochiai N. Effectiveness of bone marrow transplantation for revitalizing a severely necrotic small bone: experimental rabbit model. *J. Orthop Sci.* 2010; 15: 381-388.

[6] Barrow PC. Reproductive toxicity testing for pharmaceuticals under ICH. *Reprod Toxicol* 2009; 28:172-179.

[7] Casals JB, Pieri NC, Feitosa Ml, Ercolin AC, Roballo KC, Baretto RS, Bressan FF, Martins DS, Miglino MA, Ambrosio CE. The use of animal models for stroke research: a review. *Comp. Med* 2011; 61: 305-313.

[8] Feigin VL, Lawes CM, Bennett DA, Anderson CS. Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol*. 2003; 2: 43-53.

[9] Culp WC, Woods SD, Skinner RD, Brown AT, Lowery JD, Johnson JL, Unger EC, Hennings LJ, Borrelli MJ, Roberson PK. Dodecafluoropentane emulsion decreases infarct volume in a rabbit ischemic stroke model. *J. Vasc. Interv. Radiol*. 2012; 23: 116-121.

[10] Erdogan B, Sen O, Caner H, Ceviker N, Baykaner K, IIgit E. Intravenous and local intraarterial tissue-plasminogen activator in a rabbit model of acute thromboembolic stroke: angiographic comparison. *Adv. Ther* 2002; 19: 266-274.

[11] Liu H, Xin L, Chan BP, Teoh R, Tang BL, Tan YH. Interferon-beta administration confers a beneficial outcome in a rabbit model of thromboembolic cerebral ischemia. *Neurosci let.* 2002; 327:146-148.

[12] Burgess A, Huang Y, Waspe AC, Ganguly M, Goertz DE, Hynynen K. High-Intensity Focused Ultrsound (HIFU) for dissolution of clots in a rabbit model of embolic stroke. *PLos One* 2012; 7:e42311.

[13] Adami A, Thijs V, Tong DC, Beaulieu C, Moseley ME, Yenari MA. Use of diffusion weighted MRI to predict the occurrence and severity of hemorrhagic transformation in a rabbit model of embolic stroke. *Brain Res* 2002; 944 :32-39.

[14] Kaufman HH, Pruessner JL, Bernstein DP, Borit A, Ostrow PT, Cahall DL. A rabbit model of intracerebral hematoma. *Acta Neuropathol*. 1985; 65: 318-321.

[15] Amiridze N, Gullapalli R, Hoffamn G, Darwish R. Experimental model of brainstem stroke in rabbits via endovascular occlusion of the basilar artery. *J. Stroke Cerebrovasc* 2009; 18: 281-287.

[16] Lew SM, Gross CE, Bednar MM, Russell SJ, Fuller SP, Ellenberger CL, Howard D. Complement depletion does not reduce brain injury in a rabbit model of thromboembolic stroke. *Brain Res Bull* 1999; 48: 325-331.

[17] Johshita H, Kassell NF, Sasaki T. Blood-brain barrier disturbance following subarachnoid hemorrhage in rabbits. *Stroke*. 1990; 21: 1051-1058.

[18] Erdi MF, Guney O, Kivici A, Esen H. The effects of alpha lipoic acid on cerebral vasospasm following experimental subarachnoid hemorrhage in the rabbit. *Turk Neurosurg* 2011; 21: 527-533.

[19] Zhuang Z, Zhou ML, You WC, Zhu L, Ma CY, Sun XJ, Shi JX. Hydrogen-rich saline alleviates early brain injury via reducing oxidative stress and brain edema following experimental subarachnoid hemorrhage in rabbits. *BMC Neurosci* 2012; 13: 47.

[20] Marbacher S, Andereggen L, Neuschmelting V, Widmer HR, von Gunten M, Takala J, Jakob SM, Fandino J. A new rabbit model for the study of early brain injury after subarachnoid hemorrhage. *J. Neurosci Methods* 2012; 208: 138-145.

[21] Chen X, Wagener JF, Morgan DH, Hui L, Ghnbi O, Geiger JD. Endolysosome mechanisms associated with Alzheimer's disease-like pathology in rabbits ingesting cholesterol-enriched diet. *J. Alzheimers Dis*. 2010; 22: 1289-1303.

[22] Deci S, Lemieux SK, Smith-Bell CA, Sparks DL, Schreurs BG. Cholesterol increases ventricular volume in a rabbit model of Alzheimer's disease. *J. Alzheimers Dis* 2012; 29: 283-292.

[23] Prasanthi JR, Dasari B, Marwarha G, Larson T, Chen X, Geiger JD, Ghribi O. Caffeine protects against oxidative stress and Alzheimer's disease-like pathology in rabbit hippocampus induced by cholesterol-enriched diet. *Free Radic Biol. Med* 2010; 49: 1212-1220.

[24] Sparks DL. The early ongoing experience with the cholesterol-fed rabbit as a model of Alzheimer's disease: the old, the new and the pilot. *J. Alzheimers Dis* 2008; 15: 641-656.

[25] Sparks DL, Martin TA, Gross DR, Hunsaker JC 3rd. Link between heart disease, cholesterol, and Alzheimer's disease: a review. *Micros Res. Tech* 2000; 50:287-290.

[26] Sparks DL, Conner DJ, Browne PJ, Lopez JE, Sabbagh MN. HMG-CoA reductase inhibitors (statins) in the treatment of Alzheimer's disease and why it would be ill-advise to use one that crosses the blood-brain barrier. *J. Nutr health Aging* 2002; 6: 324-331

[27] Poole-Wilson P. The prevention of cardiovascular disease worldwide: whose task and WHO's task. *Clin Med* 2005; 5: 379-84.

[28] World Health Organization. In Fact sheet No 317 Feb 2007 (World health organization,2007)(http://www.whoint/mediacentre/factsheet/fs317/en/index.html).

[29] Getz GS, Reardon CA. Animal models of atherosclerosis. *Arterioscler Thromb. Vasc. Biol.* 2012; 32: 1104-1115.

[30] Dornas WC, Oliveir TT, Augusto LE, Nagem TJ. Experimental atherosclerosis in rabbit. *Arg Bras Cardiol* 2010; 95: 272-278.

[31] Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, Blanco-Colio L, Lavin B, Mallavia B, Tarin C, Mas S, Ortiz A, Egido J. Animal models of cardiovascular diseases. *J. Biomed Biotechnol.* 2011; 497841.

[32] Aliev G, Castellani RJ, Petersen RB, Burnstock G, Perry G, Smith MA. Pathobiology of familial hypercholesterolemic atherosclerosis. *J. Submicrosc Cytol Pathol* 2004; 36: 225-240.

[33] Kobayashi T, Ito T, Yamada S, Kuniyoshi N, Shiomi M. Electrocardiograms corresponding to the development of myocardial infarction in anesthetized WHHLMI rabbits (Oryctolagus cuniculus), an animal model for familial hypercholesterolemia. *Comp. Med* 2012; 62:409-418.

[34] Du B, Xu G, Cao H, Cui W, Lin S, Liu Y, Qin L. Effects of atorvastatin on expression of ICAM-1 in atherosclerotic rabbits. *J. Cardiovasc Med.* (Hagerstown) 2013; 14: 120-126.

[35] Tian J, Hu S, Sun Y, Yu H, Han X, Cheng W, Ban X, Zhang S, Yu B, Jang IK. Vasa vasorum and plaque progression, and responses to atorvastatin in a rabbit model of atherosclerosis: contrast-enhanced ultrasound imaging and intravascular ultrasound study. *Heart* 2013; 99:48-54.

[36] Sezer ED, Sozmen EY, Nart D, Onat T. Effect of atorvastatin therapy on oxidant-antioxidant status and atherosclerotic plaque formation. *Vasc Health Risk Manag* 2011; 7: 333-343.

[37] Yang YP, Dong QL, Zhang XH, Zhang YH, Zhu L, Li SY, Liu ZZ, Xu H, Wang N, Jiang H, Liu CX, Liu XX, Dong B. Combination of fluvastatin and losartan relieves atherosclerosis and macrophage infiltration in atherosclerotic plaques in rabbits. *Acta Pharmacol Sin* 2011;32:1259-1265.

[38] Echeverri D, Móntes F, Delgadillo A, Cabrales J. Effects of the simultaneous use of drug-eluting stents and statins in an atherosclerotic animal model. *Clin. Investig Arterioscler* 2013; 25:16-24.

[39] Davis HR Jr, Lowe RS, Neff DR Effects of ezetimibe on atherosclerosis in preclinical models. *Atherosclerosi*s 2011; 215: 266-278.

[40] Djahansouzi S, Braesen JH, Koenig K, Beisiegel U, Kontush A. The effect of pharmacological doses of different antioxidants on oxidation parameters and atherogenesis in hyperlipidaemic rabbits. *Atherosclerosis* 2001; 154: 387-398.

[41] Hope S, Brecher P, Chobanian AV. Comparison of the effects of AT1 receptor blockade and angiotensin converting enzyme inhibition on atherosclerosis. *Am. J. Hypertens* 1999; 12 : 28-34.

[42] Szeto A, Rossetti MA, Mendez AJ, Noller CM, Herderick EE, Gonzales JA, Schneiderman N, McCabe PM. Oxytocin administration attenuates atherosclerosis and inflammation in Watanabe Heritable Hyperlipidemic rabbits. *Psycho neuroendocrinology* 2013; 38: 685-693.

[43] Ichinose K, Kawasaki E, Eguchi K. Recent advancement of understanding pathogenesis of Type 1 diabetes and potential relevance to diabetic nephropathy. *Am. J. Nephrol.* 2007; 27: 554- 564.

[44] Gambelunghe G, Brozzetti A, Ghaderi M, Candeloro P, Tortoioli C, Falorni A. MICA gene polymorphism in the pathogenesis of type 1 diabetes. Ann N Y Acad Sci 2007; 1110: 92-98.

[45] Decker M, Hofflich H, Elias AN. Thiazolidinediones and the preservation of beta-cell function, cellular proliferation and apoptosis. *Diabetes Obes Metab* 2008; 10: 617-625.

[46] Plomgaard P, Nielsen AR, Fischer CP, Mortensen OH, Broholm C, Penkowa M, Krogh-Madsen R, Erikstrup C, Lindegaard B, Petersen AM, Taudorf S, Pedersen BK.

Associations between insulin resistance and TNF-alpha in plasma, skeletal muscle and adipose tissue in humans with and without type 2 diabetes. *Diabetologia* 2007; 50: 2562-2571.

[47] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes. Estimates for the year 2000 and projections for 2030. *Diabetes Care* 2004; 27: 1047-1053.

[48] Conaway HH, Brown CJ, Sanders LL, Cernosek SF, Farris HE, Roth SI. Spontaneous diabetes mellitus in the New Zealand white rabbit: history, classification, and genetic analysis. *J. Hered* 1980; 71:179-186.

[49] Thompson CS, Mumtaz FH, Khan MA, Wallis RM, Mikhailidis DP, Morgan RJ, Angelini GD, Jeremy JY. The effect of sildenafil on corpus cavernosal smooth muscle relaxation and cyclic GMP formation in the diabetic rabbit. *Eur. J. Pharmacol* 2001; 425: 57-64.

[50] Sullivan ME, Mumtaz FH, Dashwood MR, Thompson CS, Naseem KM, Bruckdorfer KR, Mikhailidis DP, Morgan RJ. Enhanced relaxation of diabetic rabbit cavernosal smooth muscle in response to nitric oxide: Potential relevance to erectile dysfunction. *Int. J. Impotence Res.* 2002; 14: 523-532.

[51] Annamala PT, Augusti KT. Studies on the biochemical effects of glibenclamide on alloxan diabetic rabbits. *Experientia* 1980; 36: 383-384.

[52] Dora CP, Singh SK, Kumar S, Datusalia AK, Deep A. Development and characterization of nanoparticles of glibenclamide by solvent displacement method. *Acta Pol. Pharm.* 2010; 67: 283-290.

[53] Habibuddin M, Daghriri HA, Humaira T, Al Qahtani MS, Hefzi AA. Antidiabetic effect of alcoholic extract of Caralluma sinaica L. on streptozotocin-induced diabetic rabbits. *J. Ethnopharmacol* 2008; 117:215-220.

[54] Nammi S, Boini MK, Lodagala SD, Behara RB. The juice of fresh leaves of Catharanthus roseus Linn. reduces blood glucose in normal and alloxan diabetic rabbits. BMC Complement *Altern Med* 2003; 3: 4.

[55] Sher A, Fakhar-ul-Mahmood M, Shah SN, Bukhsh S, Murtaza G. Effect of garlic extract on blood glucose level and lipid profile in normal and alloxan diabetic rabbits. *Adv. Clin. Exp. Med.* 2012; 21: 705-711.

[56] Thygesen K, Alpert JS, White HD. Universal definition of myocardial infarction. *Eur. Heart J*. 2007; 28: 2525-2538.

[57] Choi D, Hwang KC, Lee KY, Kim YH. Ischemic heart diseases: current treatments and future. *J. Control Release* 2009; 140: 194-202.

[58] Zhong Y, Cao P, Tong C, Li X. Effect of ramipril on the electrophysiological characteristics of ventricular myocardium after myocardial infarction in rabbits. *J. Cardiovasc. Med Hagerstown* 2012; 13: 313-318.

[59] Hu N, Sabey KH, Curtis HR, Hoang N, Dowdle SB, Garzarelli AA, Buswell HR, Dibella E, Yockman JW, Bull DA. Magnetic resonance imaging (MRI) assessment of ventricular remodeling after myocardial infarction in rabbits. *Comp. Med* 2012; 62:116-123.

[60] Ziv O, Schofield L, Lau E, Chaves L, Patel D, Jeng P, Peng X, Choi BR, Koren G. A novel, minimally invasive, segmental myocardial infarction with a clear healed infarct borderzone in rabbits. *Am. J. Physiol Heart Circ. Physiol* 2012; 302: H2321-2330.

[61] Cohen MV, Yang XM, Liu Y, Snell KS, Downey JM. A new animal model of controlled coronary artery occlusion in conscious rabbits. *Cardiovasc Res.* 1994; 28: 61-65.

[62] Katsanos K, Mitsos S, Koletsis E, Bravou V, Karnabatidis D, Kolonitsiou F, Diamantopoulos A, Dougenis D, Siablis D. Transauricular embolization of the rabbit coronary artery for experimental myocardial infarction: comparison of a minimally invasive closed-chest model with open-chest surgery. *J. Cardiothorac. Surg*. 2012; 7:16.

[63] Zhong Y, Cao P, Tong C, Li X. Effect of ramipril on the electrophysiological characteristics of ventricular myocardium after myocardial infarction in rabbits. *J. Cardiovasc. Med Hagerstown* 2012; 13: 313-318.

[64] Wang WE, Chen X, Houser SR, Zeng C. Potential of cardiac stem/progenitor cells and induced pluripotent stem cells for cardiac repair in ischaemic heart disease. *Clin. Sci.* 2013; 125: 319-327.

[65] Zhao Q, Sun C, Xu X, Zhou J, Wu Y, Tian Y, Ma A, Liu Z. Early use of granulocyte colony stimulating factor improves survival in a rabbit model of chronic myocardial ischemia. *J. Cardiol.* 2013; 61: 87-94.

[66] Manolio TA, Baughman KL, Rodeheffer R, Pearson TA, Bristow JD, Michels VV, Abelmann WH, Harlan WR. Prevalence and etiology of idiopathic dilated cardiomyopathy (summary of a National Heart, Lung, and Blood Institute workshop. *Am. J. Cardiol.* 1992; 69: 1458-1466.

[67] Matsui S, Fu ML, Katsuda S, Hayase M, Yamaguchi N, Teraoka K, Kurihara T, Takekoshi N, Murakami E, Hoebeke J, Hjalmarson A. Peptides derived from cardiovascular G-protein-coupled receptors induce morphological cardiomyopathic changes in immunized rabbits. *J. Mol Cell Cardiol.* 1997; 29: 641-655.

[68] Gava FN, Zacché E, Ortiz EM, Champion T, Bandarra MB, Vasconcelos RO, Barbosa JC, Camacho AA. Doxorubicin induced dilated cardiomyopathy in a rabbit model: an update.*Res Vet Sci*. 2013; 94: 115-121.

[69] Kamaruzaman NA, Kardia E, Kamaldin N', Latahir AZ, Yahaya BH. The rabbit as a model for studying lung disease and stem cell therapy. *Biomed. Res Int.* 2013; 2013: 691830.

[70] Shin YS, Takeda K, Gelfand EW. Understanding asthma using animal models. *Allergy, Asthma and Immunology Research*. 2009; 1: 10–18.

[71] Josephson MB, Jiao J, Xu S, Hu A, Paranjape C, Grunstein JS, Grumbach Y, Nino G, Kreiger PA, McDonough J, Grunstein MM. IL-13-induced changes in endogenous glucocorticoid metabolism in the lung regulate the proasthmatic response. *Am. J. Physiol. Lung Cell Mol Physiol*. 2012; 303: L382-390.

[72] Nino G, Hu A, Grunstein JS, McDonough J, Kreiger PA, Josephson MB, Choi JK, Grunstein MM. G Protein βγ-subunit signaling mediates airway hyperresponsiveness and inflammation in allergic asthma. *PloS One* 2012;7: e32078.

[73] Miyata R, Bai N, Vincent R, Sin DD, Van Eeden SF. Statins reduce ambient particulate matter-induced lung inflammation by promoting the clearance of particulate matter, < 10 μm from lung tissues. *Chest* 2013; 143:452-460.

[74] Mokra D, Drgova A, Kopincova J, Pullmann R, Calkovska A. Anti-inflammatory treatment in dysfunction of pulmonary surfactant in meconium-induced acute lung injury. *Adv. Exp. Med. Biol*. 2013; 756:189-196.

[75] Mokra D, Drgova A, Pullmann R Sr, Calkovska A. Selective phosphodiesterase 3 inhibitor olprinone attenuates meconium-induced oxidative lung injury. *Pulm Pharmacol Ther* 2012; 25:216-222.

[76] Baila B, Ohno Y, Nagamoto H, Kotosai K, Yabuuchi Y, Funaguchi N, Ito F, Endo J, Mori H, Takemura G, Fujiwara T, Fujiwara H, Minatoguchi S. Tetomilast attenuates elastase-induced pulmonary emphysema through inhibition of oxidative stress in rabbits. *Biol. Pharm. Bull* 2012; 35:494-502.

[77] Fioretto JR, Campos FJ, Ronchi CF, Ferreira AL, Kurokawa CS, Carpi MF, Moraes MA, Bonatto RC, Defaveri J, Yeum KJ. Effects of inhaled nitric oxide on oxidative stress and histopathological and inflammatory lung injury in a saline-lavaged rabbit model of acute lung injury. *Respir Care* 2012; 57:273-281.

[78] Roomans GM. Tissue engineering and the use of stem/progenitor cells for airway epithelium repair. *European Cells & Materials*. 2010; 19:284–299.

[79] Wetsel RA, Wang D, Calame DG. Therapeutic potential of lung epithelial progenitor cells derived from embryonic and induced pluripotent stem cells. *Annual Review of Medicine*. 2011; 62:95–105.

[80] Cao JP, He XY, Xu HT, Zou Z, Shi XY. Autologous transplantation of peripheral blood-derived circulating endothelial progenitor cells attenuates endotoxin-induced acute lung injury in rabbits by direct endothelial repair and indirect immunomodulation. *Anesthesiology* 2012;116:1278-1287.

[81] Hebbard L, George J. Animal models of nonalcoholic fatty liver disease. *Nat. Rev. Gastroenterol Hepatol* 2011; 8: 35-44.

[82] Ogawa T, Fujii H, Yoshizato K, Kawada N. A human-type non-alcoholic steatohepatitis model with advanced fibrosis in rabbits. *Am. J. Pathol*. 2010; 177: 153-165.

[83] Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis.* 2008; 28:339–350.

[84] Tuñón MJ, Alvarez M, Culebras JM, González-Gallego J. An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure. *World J. Gastroenterol.* 2009; 15: 3086–3098.

[85] Bravo E, D'Amore E, Ciaffoni F, Mammola CL. Evaluation of the spontaneous reversibility of carbon tetrachloride-induced liver cirrhosis in rabbits. *Lab Anim* 2012; 46:122-128.

[86] Jin B, Alter HJ, Zhang ZC, Shih JW, Esteban JM, Sun T, Yang YS, Qiu Q, Liu XL, Yao L, Wang HD, Cheng LF. Reversibility of experimental rabbit liver cirrhosis by portal collagenase administration. *Lab Invest* 2005; 85: 992-1002.

[87] Blitzer BL, Waggoner JG, Jones EA, Gralnick HR, Towne D, Butler J, Weise V, Kopin IJ, Walters I, Teychenne PF, Goodman DG, Berk PD. A model of fulminant hepatic failure in the rabbit. *Gastroenterology* 1978; 74: 664-671.

[88] Shang Q-L, Xiao E-H, Zhou Q-C, Luo J-G, and Wu H-J Pathological and MR-DWI study of the acute hepatic injury model after stem cell transplantation. *World J. Gastroenterol*. 2011; 17: 2821–2828.

[89] Tunon MJ, Sanchez-Campos S, Garcia-Ferreras J, Alvarez M, Jorquera F, Gonzalez-Gallego J. Rabbit hemorrhagic viral disease: characterization of a new animal model of fulminant liver failure. *J. Lab Clin. Med*. 2003; 141:272–278.

[90] Prieto JM, Fernandez F, Alvarez V, Espi A, García Marín JF, Alvarez M, Martín JM, Parra F. Immunohistochemical localisation of rabbit haemorrhagic disease virus VP-60 antigen in early infection of young and adult rabbits. *Res. Vet Sci*. 2000; 68:181–187.

[91] San-Miguel B, Alvarez M, Culebras JM, Gonzalez-Gallego J, Tunon MJ. N-acetyl-cysteine protects liver from apoptotic death in an animal model of fulminant hepatic failure. *Apoptosis.* 2006; 11:1945–1957.

[92] Tunon MJ, San Miguel B, Crespo I, Jorquera F, Santamaria E, Alvarez M, Prieto J, Gonzalez-Gallego J. Melatonin attenuates apoptotic liver damage in fulminant hepatic failure induced by the rabbit hemorrhagic disease virus. *J. Pineal Res*. 2011; 50: 38–45.

[93] Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia- reperfusion injury. *Am. J. Surg* 2001; 181:160-166.

[94] Adams JG Jr, Dhar A, Shukla SD, Silver D. Effect of pentoxifillin on tissue injury and platelet – activating factor production during ischemia–reperfusion injury. J Vasc Surg 1995; 21:742-748.

[95] Troyer-Caudle J. Reperfusion injury. *J. Vasc. Nurs* 1993; 11:76-79.

[96] Ye L, Luo CZ, McCluskey SA, Pang QY, Zhu T. Propofol attenuates hepatic ischemia/reperfusion injury in an in vivo rabbit model. *J. Surg. Res*. 2012;178: e65-70.

[97] Sheth H, Hafez T, Glantzounis GK, Seifalian AM, Fuller B, Davidson BR. Glycine maintains mitochondrial activity and bile composition following warm liver ischemia-reperfusion injury. *J. Gastroenterol Hepatol.* 2011; 26:194-200.

[98] Sanz AB, Sanchez-Niño MD, Martín-Cleary C, Ortiz A, Ramos AM. Progress in the development of animal models of acute kidney injury and its impact on drug discovery. *Expert Opin. Drug Discov*. 2013; 8: 879-895.

[99] Noone D, Licht C. Chronic kidney disease: a new look at pathogenetic mechanisms and treatment options. *Pediatr Nephrol* 2013 [Epub ahead of print].

[100] Litwin M, Niemirska A. Metabolic syndrome in children with chronic kidney disease and after renal transplantation. *Pediatr. Nephrol*. 2013 Jun 13. [Epub ahead of print]

[101] Lau DHW, Mikhailidis DP, Thompson CS. The effect of vardenafil (a PDE- 5 inhibitor) on renal function in the diabetic rabbit: a pilot study. In vivo 2007; 21: 851-854.

[102] Yakut N, Yasa H, Bahriye Lafci B, Ortac R, Tulukoglu E, Aksun M, Ozbek C, Gurbuz A. The influence of levosimendan and iloprost on renal ischemia-reperfusion: an experimental study. *Interact Cardiovasc Thorac Surg* 2008; 7: 235-239.

[103] Liu Z, Li C, Zhang Q, Tao M. Effect of Renshen polysaccharides on oxidative injury in kidney IR rabbits. *Cardohydr Polym* 2012; 90:773-777.

[104] Garcia A, Bosques CJ, Wishnok JS, Feng Y, Karalius BJ, Butterton JR, Schauer DB, Rogers AB, Fox JG. Renal injury is a consistent finding in Dutch Belted rabbits experimentally infected with enterohemorrhagic Escherichia coli. *J. Infect Dis*. 2006; 193:1125-1134.

[105] Jackson LW, Schisterman EF, Dey-Rao R, Browne R, Armstrong D. Oxidative stress and endometriosis. *Hum. Reprod* 2005; 20: 2014-2020.

[106] Yuan P, Huang Y, Wu H, Teng Z, Zhang J, Xin X. Induction of a local pseudo-pregnancy via levonorgestrel-loaded microspheres for the treatment of endometriosis in a rabbit model. *Hum. Reprod* 2010; 25: 462-469.

[107] Farquhar CM. Ectopic pregnancy. *Lancet*. 2005; 366:583–591.

[108] Popp LW, Gaetje R, Status S, Lierse W. A rabbit model for the evaluation of minimal access treatment of ectopic pregnancy in humans, using intrachorionic injection and local hyperthermia. *Clin. Exp. Obstet Gynecol* 1993; 20: 226-235.

[109] Rush RW, Keirse MJ, Howat P, Baum JD, Anderson AB, Turnbull AC. Contribution of preterm delivery to perinatal mortality. *Br. Med. J*. 1976; 2:965–968.

[110] Gorenberg D, Beharry K, Nishihara KC, Chang E, Waltzman J, AkmalA, Asrat T. Dose response of RU486 in a novel rabbit model of noninfectious preterm birth: comparative efficacy of 3 routes of administration. *Am. J. Obstet Gynecol* 2005 Mar;192: 924-931.

[111] Gibbs RS, McDuffie RS Jr, Kunze M, Barr JM, Wolf DM, Sze CI, Shikes R, Sherman MP. Experimental intrauterine infection with Prevotella bivia in New Zealand White rabbits. *Am. J. Obstet Gynecol* 2004 Apr;190: 1082-1086.

[112] Fidel P, Ghezzi F, Romero R, Chaiworapongsa T, Espinoza J, Cutright J, Wolf N, Gomez R. The effect of antibiotic therapy on intrauterine infection-induced preterm parturition in rabbits. *J. Matern Fetal Neonatal. Med*. 2003; 14: 57-64.

[113] Hasegawa A, Otsuki K, Sasaki Y, Sawada M, Mitsukawa K, Chiba H, Nagatsuka M, Okai T, Kato A. Preventive effect of recombinant human lactoferrin in a rabbit preterm delivery model. *Am. J. Obstet Gynecol.* 2005; 192: 1038-1043.

[114] Fischer B, Chavatte-Palmer P, Viebahn C, Navarrete Santos A, Duranthon V. Rabbit as a reproductive model for human health. *Reproduction* 2012; 144:1-10.

[115] Palinski W, Napoli C. The fetal origins of atherosclerosis: maternal hypercholesterolemia and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis. *FASEB J*. 2002; 16:1348-1360.

[116] Frantz E, Menezes HS, Lange KC, Abegg MP, Correa CA, Zangalli L, Vieira JL, Zettler CG. The effect of maternal hypercholesterolemia on the placenta and fetal arteries in rabbits. *Acta Cir Bras* 2012 Jan; 27:7-12.

[117] Eixarch E, Hernandez-Andrade E, Crispi F, Illa M, Torre I, Figueras F, Gratacos E. Impact on fetal mortality and cardiovascular Doppler of selective ligature of uteroplacental vessels compared with undernutrition in a rabbit model of intrauterine growth restriction. *Placenta* 2011; 32:304-309.

[118] Eixarch E, Figueras F, Hernández-Andrade E, Crispi F, Nadal A, Torre I, Oliveira S, Gratacós E. An experimental model of fetal growth restriction based on selective ligature of uteroplacental vessels in the pregnant rabbit. *Fetal Diagn Ther* 2009; 26:203-211.

[119] McArdle A, Maduwegedera D, Moritz K, Flower RL, Denton KM, Roberts CT. Chronic maternal hypertension affects placental gene expression and differentiation in rabbits. *J. Hypertens* 2010; 28:959-968.

[120] Sullivan ME, Thompson CS, Dashwood MR, Khan MA, Jeremy JY, Morgan RJ, Mikhailidis DP. Nitric oxide and penile erection: Is erectile dysfunction another manifestation of vascular disease? *Cardiovascular Res*.1999; 43: 658-665.

[121] Bredt DS, Snyder SH. Nitric oxide: a physiological messenger molecule. *Annu. Rev. Biochem*. 1994; 63: 175-195.

[122] Trigo-Rocha F, Hsu GL, Donatucci CF, Lue TF: The role of cyclic adenosine monophosphate, cyclic guanosine monophosphate, endothelium and nonadrenergic, noncholinergic , neurotransmission in canine penile erection. *J. Urol.* 1993; 149: 872-877.

[123] Droggrell SA: Comparison of clinical trials with sildenafil, vardenafil and tadalafil in erectile dysfunction. *Expert Opin. Pharmacother* 2005; 6: 75-84.

[124] Jackson G, Rosen RC, Kloner RA, Kostis JB. The second Princeton consensus on sexual dysfunction and cardiac risk: new guidelines for sexual medicine. *J. Sex Med.* 2006; 3: 28-36.

[125] Bischoff E. Rabbits as models for impotence research. *Int. J. Impot Res.* 2001; 13 :146-148.

[126] Stief CG, Benard F, Bosch RJLH, Aboseif SR, Tanagho EA. The rabbit as a model for neurourologic studies of the lower genitourinary tract. *World J. Urol.* 1990; 8: 233-236.

[127] Choi S, Min K, Kim NN, Munarriz R, Goldstein I, Traish AM. Laser oximetry: A novel noninvasive method to determine changes in penile hemodynamics in an anesthetized rabbit model. *J. Androl*. 2002; 23: 278-283.

[128] Azadzoi KM, Park K, Andry C, Goldstein I, Siroky MB. Relationship between cavernosal ischemia and corporal veno-occlusive dysfunction in an animal model. *J. Urol.* 1997;157:1011-1017.

[129] Bischoff E, Schneider K. A conscious-rabbit model to study vardenafil hydrochloride and other agents that influence penile erection. *In. J. Impot Res*. 2001; 13: 230-235.

[130] Firoozi F, Longhurst PA, White MD. In vivo and in vitro responses of corpus cavernosum to phosphodiesterase-5 inhibition in the hypercholesterolaemic rabbit. *BJU Int.* 2005; 96: 164-168.

[131] Ahn BO, Kang KK, Ahn GJ, Kwon JW, Kim WB, Kang KS, Lee YS. Efficacy of DA-8159, a new PDE5 inhibitor, for inducing penile erection in rabbits with acute spinal cord injury. *Int. J. Impot Res.* 2003; 15:405-411.

[132] Lerner SE, Melman A, Christ GJ: A review of erectile dysfunction: new insights and more questions. *J. Urol*. 1993; 149: 1246-1255.

[133] Ziegler D, Merfort F, van Ahlen H, Yassin A, Reblin T, Neureither M: Efficacy and safety of flexible-dose vardenafil in men with type 1 diabetes and erectile dysfunction. *J. Sex Med*. 2006; 3: 883-891.

[134] Thompson CS, Mumtaz FH, Khan MA, Wallis RM, Mikhailidis DP, Morgan RJ, Angelini GD, Jeremy JY. The effect of sildenafil on corpus cavernosal smooth muscle relaxation and cyclic GMP formation in the diabetic rabbit. *Eur. J. Pharmacol* 2001; 425: 57-64.

[135] Lau DHW, Mumtaz FH, Mikhailidis DP, Thompson CS. The in vitro and in vivo effects of vardenafil (a PDE-5 inhibitor) on corpus cavernosal smooth muscle relaxation in diabetic rabbits. *Urol Int* 2009; 82: 101-107.

[136] Berry SJ, Coffey DS, Walsh PC, Ewing LL. The development of human benign prostatic hyperplasia with age. *J. Urol* 1984; 132: 474-479.

[137] Namasivayam S, Minhas S, Brooke J, Joyce AD, Prescott S, Eardley I. The evaluation of sexual function in men presenting with symptomatic benign prostatic hyperplasia. Br *J. Urol.* 1998; 82: 842-846.

[138] Baniel J, Israilov S, Shmueli J, Segenreich E, Livne PM. Sexual function in 131 patients with benign prostatic hyperplasia before prostatectomy. *Eur. Urol*. 2000; 38: 53-58.

[139] Rosen RC, Wei JT, Althof SE, Seftel AD, Miner M, Pereiman MA. Association of sexual dysfunction with lower urinary tract symptoms of BPH and BPH medical therapies: results from the BPH registry. *Urol* 2009; 73: 562-566.

[140] Calvert RC, Thompson CS, Khan MA, Mikhailidis DP,Morgan RJ, Burnstock G. Alterations in cholinergic and purinergic signalling in a model of the obstructed bladder. *J. Urol*. 2001; 166: 1530-1533.

[141] Lin W-Y, Mannikarottu A, Chichester P, Neuman P, Johnson A, Perez-Martinez FC, Levin RM. The effect of chronic partial bladder outlet obstruction on corpus cavernosum smooth muscle and rho-kinase in rabbits. *Neurourology and Urodynamics* 2008; 27: 826-831.

[142] Ertemi H, Lau DHW, Mikhailidis DP, Mumtaz FH, Thompson CS. Angiotensin II increases corpus cavernosal contractility and oxidative stress in partial bladder outlet obstructed rabbits: relevance to erectile dysfunction. *J. Sex Med*. 2013; 10: 1251-1258.

[143] Das S, Crockett JC. Osteoporosis - a current view of pharmacological prevention and treatment. *Drug Des Devel Ther*. 2013; 7:435-448.

[144] Almagro MI, Roman-Blas JA, Bellido M, Castañeda S, Cortez R, Herrero-Beaumont G. PTH [1-34] enhances bone response around titanium implants in a rabbit model of osteoporosis. *Clin. Oral Implant Res* 2012 [Epub ahead of print].

[145] Carvas JS, Pereira RM, Caparbo VF, Fuller P, Silveira CA, Lima LA, Bonfa E, Mello SB. A single dose of zoledronic acid reverses the deleterious effects of glucocorticoids on titanium implant osseointegration. *Osteoporos Int* 2010; 21:1723-1729.

[146] Zhang KJ, Zhang J, Kang ZK, Xue XM, Kang JF, Li YW, Dong HN, Liu DG. Ibandronate for prevention and treatment of glucocorticoid-induced osteoporosis in rabbits. *Rheumatol. Int* 2012; 32:3405-3411.

[147] Li GY, Yin JM, Ding H, Jia WT, Zhang CQ. Efficacy of leukocyte- and platelet-rich plasma gel (L-PRP gel) in treating osteomyelitis in a rabbit model. *J. Orthop Res*. 2013; 31:949-956.

[148] Beenken KE, Bradney L, Bellamy W, Skinner RA, McLaren SG, Gruenwald MJ, Spencer HJ, Smith JK, Haggard WO, Smeltzer MS. Use of xylitol to enhance the therapeutic efficacy of polymethylmethacrylate-based antibiotic therapy in treatment of chronic osteomyelitis. *Antimicrob agents Chemother* 2012; 56:5839-5844.

[149] Wu X, Yang S, Wang H, Meng C, Xu W, Duan D, Liu X. G-CSF/SCF exert beneficial effects via anti-apoptosis in rabbits with steroid-associated osteonecrosis. *Exp. Mol. Pathol*. 2013; 94 :247-254.

[150] Wen Q, Jin D, Zhou CY, Zhou MQ, Luo W, Ma L. HGF-transgenic MSCs can improve the effects of tissue self-repair in a rabbit model of traumatic osteonecrosis of the femoral head. *PLosOn*e 2012; 7: e37503.

[151] Xie XH, Wang XL, He YX, Liu Z, Sheng H, Zhang G, Qin L. Promotion of bone repair by implantation of cryopreserved bone marrow-derived mononuclear cells in a rabbit model of steroid-associated osteonecrosis. *Arthritis Rheum* 2012; 64:1562-1571.

[152] Nalca A, Nichols DK. Rabbitpox: a model of airborne transmission of smallpox. *J. Gen. Virol* 2011; 92:31-35.

[153] Nalca A, Hatkin JM, Garza NL, Nichols DK, Hurby DE, Jordan R. Evaluation of orally delivered ST-246 as postexposure prophylactic and antiviral therapeutic in an aerosolized rabbitpox rabbit model. *Antiviral Res* 2008; 79: 121–127.

[154] Garza NL, Hatkin JM, Nichols DK, Livingston V, Nalca A. Evaluation of efficacy of modified vaccinia virus (MVA) vaccine against aerosolized rabbitpox virus. *Vaccine* 2009; 27: 5496–5504.

[155] Rice AD, Adams MM, Wallace G, Burrage AM, Lindsey SF, Smith AJ, Swetnam D, Manning BR, Gray SA, Lampert B, Foster S, Lanier R, Robertson A, Painter G, Moyer RW. Efficacy of CMX001 as a post exposure antiviral in New Zealand White rabbits infected with rabbitpox virus, a model for orthopoxvirus infections of humans. *Viruses* 2011; 3:47-62.

[156] Crickard L, Babas T, Seth S, Silvera P, Koriazova L, Crotty S. Protection of rabbits and immunodeficient mice against lethal poxvirus infections by human monoclonal antibodies. *PlosOne* 2012; 7: e48706.

[157] Cheng X, Wang S, Dai X, Shi C, Wen Y, Zhu M, Zhan S, Meng J. Rabbit as a novel animal model for hepatitis E virus infection and vaccine evaluation. *PlosOne* 2012; 7: e51616.

[158] Wu X, Tao P, Nie H. Geldanamycin is effective in the treatment of herpes simplex virus epithelial keratitis in a rabbit model. *Clin. Experiemtal. Ophthalmol.* 2011; 39: 779-783.

[159] Clement C, Capriotti JA, Kumar M, Hobden JA, Foster TP, Bhattacharjee PS, Thompson HW, Mahmud R, Liang B, Hill JM. Clinical and antiviral efficacy of an ophthalmic formulation of dexamethasone povidone-iodine in a rabbit model of adenoviral keratoconjunctivitis. *Invest Ophthalmol Vis Sci* 201; 52:339-344.

[160] Brousseau ME, Hoeg JM. Transgenic rabbits as models for atherosclerosis. *J. Lipid Res*. 1999; 40:365-375.

[161] Hiripi L, Negre D, Cosset FL, Kvell K, Czömpöly T, Baranyi M, Gócza E, Hoffmann O, Bender B, Bosze Z. Transgenic rabbit production with simian immunodeficiency virus-derived lentiviral vector. *Transgenic Res* 2010; 19:799-808.

[162] Peng X. Transgenic rabbit models for studying human cardiovascular diseases. *Comp Med.* 2012; 62:472-479.

[163] Koike T, Kitajima S, Yu Y, Li Y, Nishijima K, Liu E, Sun H, Waqar AB, Shibata N, Inoue T, Wang Y, Zhang B, Kobayashi J, Morimoto M, Saku K, Watanabe T, Fan J. Expression of human apoAII in transgenic rabbits leads to dyslipidemia: a new model for combined hyperlipidemia. *Arterioscler Thromb Vasc Biol*. 2009; 29:2047-2053.

[164] Wang Y, Niimi M, Nishijima K, Waqar AB, Yu Y, Koike T, Kitajima S, Liu E, Inoue T, Kohashi M, Keyamura Y, Yoshikawa T, Zhang J, Ma L, Zha X, Watanabe T, Asada Y, Chen YE, Fan J. Human apolipoprotein A-II protects against diet-induced atherosclerosis in transgenic rabbits. *Arterioscler. Thromb Vas. Biol*. 2013; 33:224-231.

[165] Ding Y, Wang Y, Zhu H, Fan J, Yu L, Liu G, Liu E. Hypertriglyceridemia and delayed clearance of fat load in transgenic rabbits expressing human apolipoprotein CIII. *Transgenic Res* 2011; 20 :867-875.

[166] Brunner M, Peng X, Liu GX, Ren XQ, Ziv O, Choi BR, Mathur R, Hajjiri M, Odening KE, Steinberg E, Folco EJ, Pringa E, Centracchio J, Macharzina RR, Donahay T, Schofield L, Rana N, Kirk M, Mitchell GF, Poppas A, Zehender M, Koren G. Mechanisms of cardiac arrhythmias and sudden death in transgenic rabbits with long QT syndrome. *J. Clin. Invest* 2008; 118: 2246-2259.

[167] Odening KE, Hyder O, Chaves L, Schofield L, Brunner M, Kirk M, Zehender M, Peng X, Koren G. 2008. Pharmacogenomics of anesthetic drugs in transgenic LQT1 and LQT2 rabbits reveal genotype-specific differential effects on cardiac repolarization. *Am. J. Physiol Heart Circ. Physiol.* 295:H2264–H2272.

[168] Bentzen BH, Bahrke S, Wu K, Larsen AP, Odening KE, Franke G, vans Gravesande KS, Biermann J, Peng X, Koren G, Zehender M, Bode C, Grunnet M, Brunner M. Pharmacological activation of Kv11.1 in transgenic long QT1 rabbits. *J. Cardiovasc Pharmacol*. 2011; 57:223–230.

[169] Biermann J, Wu K, Odening KE, Asbach S, Koren G, Peng X, Zehender M, Bode C, Brunner M. Nicorandil normalizes prolonged repolarisation in the first transgenic rabbit model with long QT syndrome 1 both in vitro and in vivo. *Eur. J. Pharmacol* 2011; 650: 309–316.

[170] Stanley BA, Graham DR, James J, Mitsak M, Tarwater PM, Robbins J, Van Eyk JE. Altered myofilament stoichiometry in response to heart failure in a cardioprotective α-myosin heavy chain transgenic rabbit model. *Proteomics Clin. Appl.* 2011; 5:147-158.

[171] Kondo M, Sakai T, Komeima K, Kurimoto Y, Ueno S, Nishizawa Y, Usukura J, Fujikado T, Tano Y, Terasaki H. Generation of a transgenic rabbit model of retinal degeneration. *Invest Ophthalmol Vis. Sci*. 2009; 50:1371-1377.

[172] Morimoto T, Kanda H, Kondo M, Terasaki H, Nishida K, Fujikado T. Transcorneal electrical stimulation promotes survival of photoreceptors and improves retinal function in rhodopsin P347L transgenic rabbits. *Invest Ophthalmol. Vis.* Sci 2012; 53: 4254-4261.

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter V

The Assessment of Sperm DNA Damage in the Rabbit Using the Halomax Assay

J. Gosálvez[1], C. López-Fernández[1], F. Arroyo[1], A. Gosálbez[1], E. I. Gutiérrez-Cortés[2] and S. D. Johnston[3]

[1]Faculty of Biology, Autonomous University of Madrid, Cantoblanco, Madrid, Spain
[2]Department of Genetics, Nothwest Research Center of Biology, Mexican National Institute for Health, Monterrey, Mexico
[3]School of Agriculture and Food Science. The University of Queensland, Gatton, Australia

Abstract

While it may be possible to obtain a broad view of semen quality from the rabbit ejaculate using assessments of sperm concentration, motility and morphology, none of these estimates provide information about the integrity of sperm chromatin, which in other species, has proven to be an interesting new approach for gaining insight about the semen sample with regards to its potential for successful fertilization and embryonic development. This study was conducted to evaluate a sperm DNA fragmentation assessment methodology based on the capacity of controlled protein depletion (Halomax-assay) to assess rabbit SDF and which was validated against indirect (comet assay) and direct (*in situ* nick-translation) measures of sperm DNA damage. There was a high degree of correlation between estimates of sperm DNA fragmentation using the Halomax and the neutral comet assay (r^2 = 0.964; $p<0.001$); there was also a direct correlation between Halomax assay and the results obtained from DNA labelling using forced polymerase nucleotide incorporation by means of the *in situ* nick translation assay. The validated Halomax assay was then used to determine the baseline level of SDF and sperm DNA longevity from 63 HYLA commercial breed rabbits and calculated as the rate of SDF degradation (r-SDF) following incubation at 38 ºC for 7h. The mean (± SEM) baseline level of SDF obtained after ejaculation was 9.1 ± 1.2 %. The mean (± SEM) rate of SDF (rSDF) obtained after 7h of incubation was 2.62 ± 0.34% per h. Differences in the baseline level of SDF and in the r-SDF were found among different animals. The procedures outlined in this study now allow SDF measurement to be implemented in

rabbit AI centres as a routine assay to select for high quality semen doses with reduced levels of chromatin damage after ejaculation and which show a low rate of DNA damage following incubation in an environment that mimics the female reproductive tract.

Keywords: Reproduction in rabbits; male factor; sperm DNA fragmentation; Sperm chromatin dispersion

Introduction

In this era of assisted reproductive technology, the concept of the spermatozoon as an important carrier of valuable inheritable information has typically not received the required attention. For each ejaculate, the andrologist routinely evaluates sperm parameters such as motion characteristics, membrane quality, morphology and concentration in order to acquire information about the potential capacity for fertility, but more often than not, the contribution of damaged DNA in the ejaculate is not assessed. This lack of attention to evaluating the quality of sperm chromatin and the state of the deoxyribonucleic acid molecule, the so called sperm DNA fragmentation (SDF), is no doubt, one of the primary reasons why pregnancy rates achieved after the application of artificial reproduction techniques (ART) is not closer to 100%. Particularly in domestic animal production, there has been only marginal interest given to the integrity of the DNA molecule despite its critical role for syngamy and subsequent embryonic development and pregnancy and yet different research groups have consistently shown that SDF is of high importance for reproductive outcome (Lopes et al., 1998; Evenson et al., 1999; Agarwal and Allamaneni 2004; Zini and Libman, 2006; Shafik et al., 2006; Bungum et al., 2007; Bungum 2012). We argue that the assessment of sperm DNA integrity should be included as part of the standard seminogram and to ignore the putative relevance of SDF on pregnancy is to ignore a major contributor towards male factor infertility and in the case of animal production, leads to a waste of resources in maintaining low efficient animals for the purposes they have been acquired.

Our research group first published SDF assessment for boars in a commercial piggery and were greatly surprised by the incidence of an unusually high level of SDF in commercial straws, which occurred in approximately 15% of the boars (López-Fernández et al. 2008a). Although the measurement of SDF in domestic animals is gaining increasing acceptance, studies determining sperm DNA damage in rabbits are limited (Gogel et al. 2000; 2002) and only based on the sperm chromatin structure assay optimised for human spermatozoa (Evenson, 1991). The Sperm Chromatin Structure Assay (SCSA) was one of the first strategies developed to assess SDF (Evenson, 1991). This methodology exploits a very simple rationale, in which a mild acidic solution is exposed to the native chromatin, resulting in the denaturation of putative double-stranded or single-stranded breaks existing in the sperm DNA and the consequent formation of single strand stretches of DNA. These single stranded stretches are then stained with the DNA binding fluorochrome acridine orange that fluoresces green with double-stranded non-denatured DNA and red with single-stranded denatured DNA, thereby allowing for the quantification of the population of sperm cells with fragmented DNA using a flow cytometer or by fluorescence microscopy. Another approach that has been successfully implemented to assess sperm DNA breakage is based upon the enzymatic addition of labelled nucleotides to the ends of an existing DNA break and includes

the proven techniques of terminal deoxynucleotidyl transferase (TdT)-mediated nick end labelling (TUNEL) or *in situ* nick translation (ISNT) using *E. coli* DNA polymerase (Domínguez-Fandos et al., 2007; Gosálvez et al., 2011). The comet assay, with different variants such as the neutral comet (N-Comet), alkaline comet (Fernández et al., 2001) or two dimensional comets (Enciso et al., 2009) as a combination of both strategies, has also been demonstrated to be an efficient means of detecting sperm DNA damage. These comet assays consist of performing single-cell gel electrophoresis on spermatozoa trapped on microgels; because of the differential resistance encountered by DNA molecules of different sizes when moving through the gel, a characteristic "comet" distribution is formed after fluorescent staining, with a dense head containing long molecules of DNA and a tail of varying length with shorter fragments of DNA. Thus, DNA breakage can be evaluated by measuring the number of cells with "migration tails". The length of the tail and/or percentage of DNA contained in the tail can then be correlated with the degree of DNA damage.

Another method for sperm DNA fragmentation and the one that we shall explore in this chapter is the Sperm Chromatin Dispersion (SCD) test (Fernández et al., 2009; Gosálvez et al., 2011a). Originally designed for use with human spermatozoa, this procedure has now been applied and validated across a wide range of species from invertebrates to domestic animals and wildlife (Cortés-Gutiérrez et al., 2008; Portas et al., 2009; Zee et al., 2009a). This methodology is based on a controlled DNA denaturation and protein depletion, which finally produces different sperm morphologies that can be correlated with presence of DNA damage. In the case of non-primate species, this procedure gives rise to haloes of chromatin dispersion due to the spreading of nuclear DNA loops and/or fragments of DNA when the spermatozoon contains fragmented DNA. The size of the halo produced after massive protein depletion is related to the amount of sperm DNA damage contained in the spermatozoa (Zee et al., 2009b). One of the major limitations for a widespread use of SDF assessment across different species is the varying nature of the para-crystaline structure of the sperm chromatin, which renders the DNA molecule highly compacted and protected, making it difficult to assess the actual state of the DNA molecule directly without some form of chemically induced change to the structure of the DNA molecule. Given that the protein of the sperm cell differs between different species, the procedure for protein depletion is therefore typically likely to be species dependent, so that the SCD needs to be co-validated with other methodologies such as ISNT and Comets. The aim of the present investigation was to evaluate a commercial version of Halomax assay adapted for rabbit spermatozoa and validate the technique against direct incorporation of labelled nucleotides on DNA breaks using ISNT and the Neutral-Comet assay (N-Comet). Additionally, we used the SCD to determine the prevalence of SDF in a commercial rabbit population and examine the longevity of rabbit sperm DNA following incubation at body temperature to test for differences among individuals.

Material and Methods

Animals and Semen Collection

This study utilized HYLA commercial breed rabbits that were randomly selected from a population resident in a commercial Spanish rabbit-breeding centre. Semen samples were

collected using an artificial vagina (Amantea; IMV, Technologies, France) from 81 sexually mature males during the peak of the breeding season in spring. Semen from 18 animals was used to conduct the initial validation studies of a modified Halomax assay by means of direct (*In situ* Nick Translation -ISNT) and indirect (Neutral Comet assay -N-Comet-) comparative evaluations. Semen samples from the remaining 63 rabbits were then used to determine the baseline level of DNA fragmentation with the breeding facility and examine sperm DNA dynamic loss of DNA quality following extended incubation at 38 ºC.

The Halomax Assay

The degree of DNA damage in each sample was quantified using a commercial version of the sperm chromatin dispersion test (Halomax, Halotech SL Madrid, Spain). The Halomax assay has previously been used in a range of mammalian species such as boar, stallion, bull, ram, elephants or cervids (Gosálvez et al., 2011b), but never used to assess SDF in rabbits in a large sample. Ejaculate volume ranged from 0.3 to 0.6 ml, but given that the sperm concentration is typically high (150 to 500 × 10^6 mL^{-1}), all semen samples were adjusted to a concentration of 10 x 10^6 mL^{-1} using RPMI Media 1640 (Life Technologies; Carlsbad, California, US). A volume of 25 μL of diluted sample was added to a vial containing low melting point agarose at 37 ºC and gently mixed. The agarose-sperm mixture (10 μL) was then prepared on pre-treated slides provided in the kit and covered with a glass coverslip. After gently pressing on the coverslip, each slide was placed in a refrigerator for 5 min to produce a microgel; once formed, the coverslip was removed from the microgel and the slide placed horizontally in 10 mL of the lysing solution provided in the kit for 5 min to achieve controlled protein depletion. The treated microgel containing partially de-proteinized sperm was then subsequently washed in dH_2O for 5 min and dehydrated in a series of ethanol baths (70%, 90% and 100%.). Once dehydrated, sperm were stained with the DNA binding fluorochrome DAPI (D9542 Sigma-Aldrich; St. Louis, MO, USA) using a stock prepared at 10% and diluted 1:10 with an anti-fading solution at the time of analysis under the microscope.

In Situ Nick Translation Assay

Direct validation between the morphology of the sperm processed using the Halomax assay and the actual presence of DNA fragmentation was performed by direct incorporation of labelled nucleotides using i*n situ* nick translation, according to the methodology previously described for boar sperm (Enciso et al., 2006); only chromatin containing DNA damage incorporates the labelled nucleotides after the ISNT assay and the morphology of the labelled spermatozoa can be directly compared with the morphology obtained after the Halomax assay. For this purpose, spermatozoa were loaded into microgels and processed as per the Halomax assay but not including the dehydration step. Once protein depletion was completed, the slide was washed in phosphate buffer saline for 10 min. Following this washing step the microgel was not allowed to dry out and the slide further incubated for 5 min in the buffer used for ISNT but without DNA polymerase. The buffer was then removed and 100 μL of the polymerase reaction buffer containing 25 units of DNA-polymerase-I (New England BioLabs, Beverly, MA, USA) and biotin-16-dUTP in the nucleotide mix, was directly pipetted onto the slide, covered with a plastic coverslip and incubated in a humidified chamber for 5, 20, and 30 min at 37 ºC to compare the level of DNA labelling on each

spermatozoon. After washing in a Tris-buffer (TBE; pH 8; 0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA) the slides were dehydrated in sequential 70% - 90% - 100% ethanol baths and air-dried. The incorporated biotin-16-dUTP was detected after incubation for 30 min with avidin conjugated with fluorescein isothiocyanate (Thermo Scientific; Rockford, IL, USA). The sperm in the microgels were counterstained using propidium iodide (2 mg/mL) in Vectashield (Vector, Burlingame, CA, USA). As a control, a designated area of the slide was incubated with the reaction buffer alone, omitting the DNA-polymerase I. Control experiments were performed on the same slide by constructing a physical barrier (gel scratched off slide) between areas of the microgel exposed with and without polymerase to avoid possible diffusion of the enzyme. Following ISNT, spermatozoa with varying degrees of chromatin damage were digitally photographed at 600x magnification and the proportion of nuclei that stained positively with the DNA label determined. The ISNT unlabelled spermatozoa typically stained only red if the nucleotides were not incorporated but green if DNA damage was present (see Figure 2).

Comet Assay

Indirect evaluation on the ability of the Halomax-assay to assess SDF was validated using the N-Comet assay. The assay was conducted on microgel slides that were treated with the same lysis solution as those used for the Halomax-assay for 5 min. The lysis solution was removed using a 1xTBE buffer solution for 5 min and the processed microgels electrophoresed using 1xTBE buffer (12 min; 20 V); DNA fragments resulting from double-stranded DNA breaks migrated from the nucleus toward the anode. The slide was then removed from the electrophoretic tray and placed in 0.9% sodium chloride solution for 2 min, followed by 5 min in 0.4 M Tris-hydrochloride buffer (pH 7.5) and 2 min in 1xTBE; finally an ethanol series (70%, 90%, and 100%) was used to dehydrate the microgels. For visualization of the DNA comet, the slides were stained with propidium iodide and viewed under a fluorescence microscope. At least 300 cells were analyzed for each individual semen sample using a 40X objective. A Pearson correlation of the frequencies of SDF observed after the slides were processed with Halomax and the level of DNA damage determined by the N-Comet assay was conducted.

Dynamics of DNA Fragmentation

In order to determine the sperm DNA longevity, aliquots of each diluted sample were incubated at 38 ºC using RPMI media. SDF (proportion of sperm with fragmented DNA) was assessed after incubation for 0h, 2h and 7h. Replicates of the same semen samples were used as internal control for each ejaculate; estimates obtained for each time period were not to be significantly different from each other ($p<0.05$). A replicate of each sperm sample during the dynamic assessment of sperm DNA damage is important to ensure that results are not compromised by problems presented by microgel detachment or slide processing failure.

Image Capture and Analysis

All slides were analyzed using a Leica DMRB (Leica Microsystems, Wetzlar, Germany) epifluorescence microscope using 40x or 60x magnification Plan-Fluotar lenses. Single-band fluorescence block filters (Semrock, Rochester, USA) for green and red fluorescence visualization were used. Images were captured using a cooled CCD DFC-350-FX Leica for

16-bit gray-level imaging. In the case of ISNT, where simultaneous fluorescence visualization using two channels was performed, each captured image was filed as separated channels in gray level; image merging was performed with Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA, USA). Colour image construction using gray-level images were performed using channel assignation merging within Adobe Photoshop. Image analysis was performed to assess the differences of DNA labelling observed among the different spermatozoa after ISNT. For this purpose, integrated density (a correlation between the area in pixels and the value associated to each pixel) was obtained on each sperm after DNA labelling. The values among 3000 randomly scanned sperm nuclei were compared after background subtraction and automatic threshold to select the region of interest; in this case the green signal obtained after ISNT. Integrated density calculations were performed using Image J free software for image analysis (NIH, Bethesda, Maryland, USA).

Statistical Analysis

SPSS 19 software package for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and graphic production. Correlation analysis was assesses using a non-parametric Pearson correlation. Differences in dynamic behaviour of SDF where analysed as survival curves using the Kaplan-Meier estimator statistic; curves were compared using the log-rank test. Significance was defined as $p < 0.05$.

Results

Halomax Assay

Semen samples processed with the Halomax assay resulted in two fundamentally different sperm morphotypes; sperm nuclei that displayed small compact haloes of chromatin dispersion (basal haloes of dispersed chromatin) were regarded as those with unfragmented DNA (Figure 1a), while nuclei showing evidence of moderate (Figure 1b) or large haloes (Figure 1c) were interpreted as possessing fragmented DNA. A general microscope field of view documenting spermatozoa with and without haloes of dispersed chromatin after fluorescence staining is showed in Figure 1d.

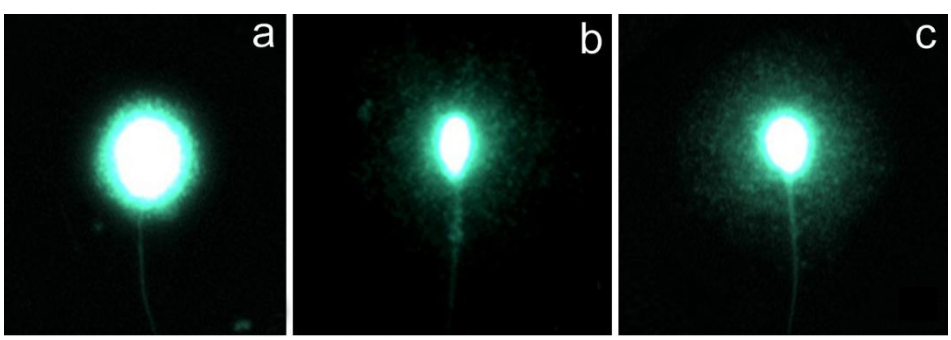


Figure 1. (Continued).

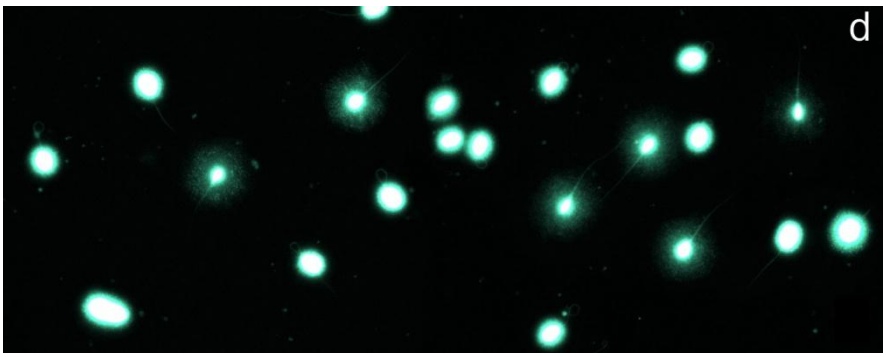


Figure 1. Sperm DNA damage visualized after the Halomax assay. Sperm nuclei that displayed small compact haloes of chromatin dispersion corresponded to spermatozoa with unfragmented DNA (a); Spermatozoa showing evidence of moderate (b) or large haloes (c) were interpreted as spermatozoa presenting fragmented DNA. Panel (d) shows a general microscope field with spermatozoa with and without haloes of dispersed chromatin.

Direct Visualization of SDF after the *In Situ* Nick Translation Assay

The optimal experimental conditions to visualize DNA labelling in order to produce a low level of unspecific background staining occurred after 30 min of polymerase extension. Using this protocol, there was direct correspondence between enzymatic incorporation of modified nucleotides and those sperm heads that showed haloes of chromatin dispersion irrespective of the size of the halo exhibited after protein depletion (Compare Figure 2a "Halomax assay" with Figure 2b and c "ISNT assay"). Using image analysis, the background signal obtained in spermatozoa showing absence of haloes after *in situ* polymerase DNA extension (sperm containing non fragmented DNA) was in the order of 80 % lower than the values obtained in those spermatozoa with a halo of dispersed chromatin (sperm containing fragmented DNA). The halo size observed in some spermatozoa with a small and residual core was up to double the size of the halos observed after direct fluorescence DNA labelling (Figure 2c). In general, the larger the size of the halo the smaller was the nucleoid core (compare Figure 2b with Figure 2c).

Correlation between the N-Comet and the Halomax Assay

After treating spermatozoa with the Halomax assay or with the N-Comet assay, both techniques showed similar morphological evidence associated with the sperm DNA damage, especially when the morphology of the basal haloes was compared (compare Figure 2d and b with 2e and f). While the basal halo is the manifestation of partial protein depletion without evidence of DNA damage, these haloes are also observed after the N-Comet assay (compare arrowed spermatozoa in Figure 2e and f). In the case of the Halomax assay, DNA fragments emerging from a remnant central core and visualized as a large halo of dispersed chromatin (Figure 2a and d) are equivalent to the fragmented DNA in the tail of the comet (Figure 2e and f).

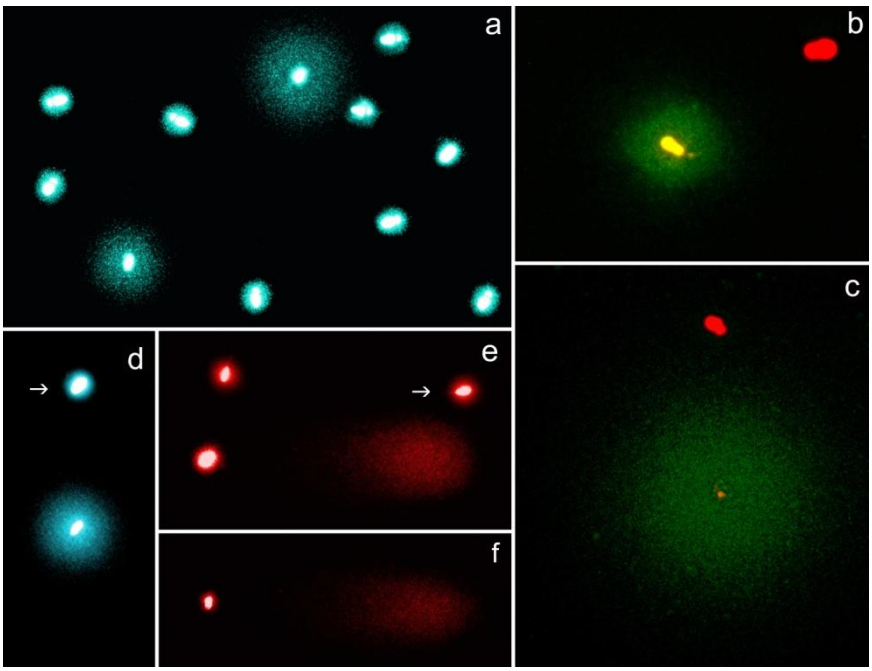


Figure 2. Visualization of sperm DNA damage in rabbits using the Halomax assay (a and d), *In situ* nick translation (b and c) and the N-Comet assay (e and f).

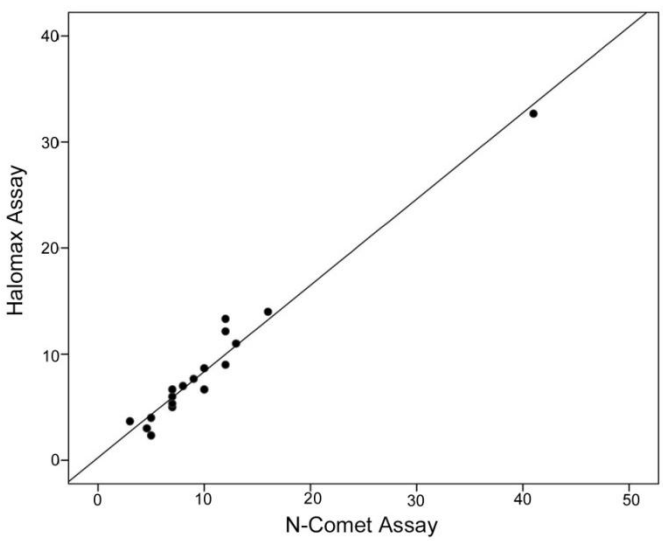


Figure 3. Correlation between sperm DNA fragmentation coincidently assessed with the Halomax and N-Comet assays.

In the N-Comet assay, a reduction in the core is also observed as the intensity of the DNA damage increases and corresponds to the DNA fragments localized in the tail which tends to be less visible and separated from the remnant nucleoid core. In order to determine whether the Halomax assay was detecting the same form of DNA damage as that detected in the N-

Comet assay, a further validation was performed using 16 different sperm samples that were simultaneously processed with both techniques; there was a strong correlation between the SDF values obtained with the two techniques (Figure 3; Pearson correlation; $r^2 = 0.964$; $p<0.001$).

Baseline and Dynamic Assessment of Sperm DNA Quality Loss

The raw data for each individual and frequency distribution of the values for SDF observed for all 63 rabbits is documented in figure 4. The mean (± SD) baseline level using the Halomax assay was 9.2% ± 9.5%. The distribution of the SDF values did not fit a normal distribution and only 5 % of the sperm samples exhibited levels of SDF higher than 15% and can, therefore, be considered out of the normal distribution (Figure 4b).

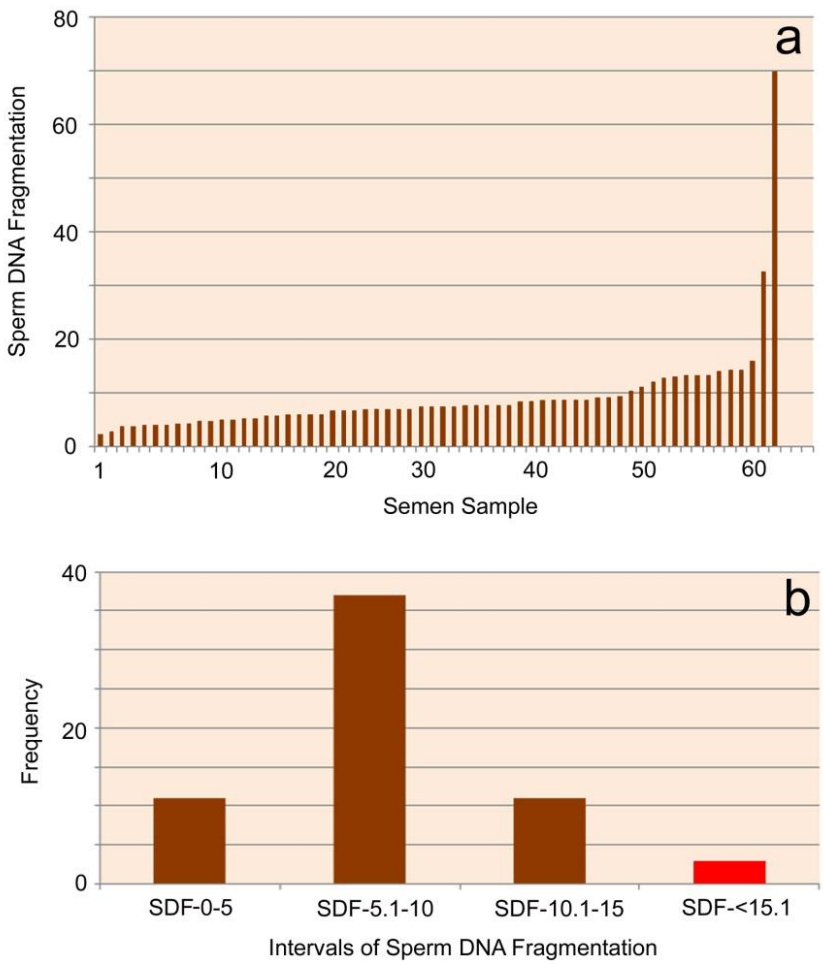


Figure 4. Net SDF values observed in 63 individual semen samples (Figure 4a) and the corresponding frequency distribution. SDF value ranges were established from 0 - 5%, from 5.1 - 10%, from 10.1 - 15% or higher than 15.1%.

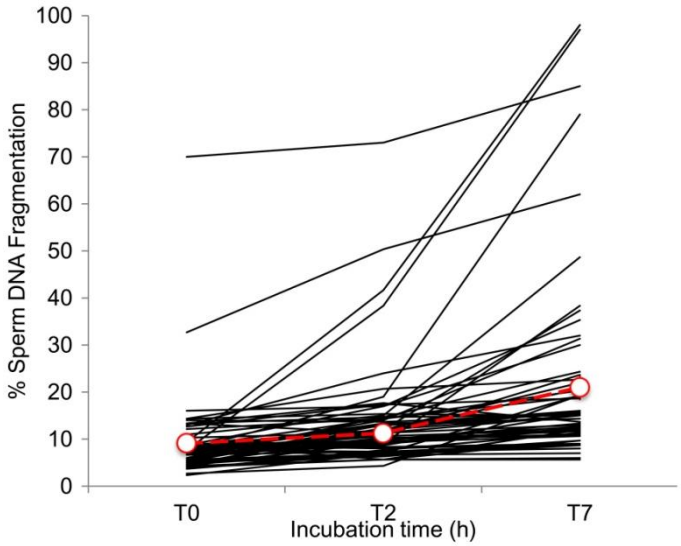


Figure 5. Rabbit sperm DNA fragmentation dynamics after incubation at 38 °C for 7h. The mean for all the SDF values at each time interval is highlighted in a dashed red line.

Sperm DNA longevity in the same semen samples was also assessed over an incubation period of 7h at 38 ºC to calculate the r-SDF. The mean (± SEM) r-SDF was 1.79% ± 0.34 of DNA damage per hour (Figure 5). Differences in the rate of SDF among different samples, taking into account the sperm DNA longevity, was assessed using a maximum likelihood estimator (Kaplan Meier) to compare the survival rate; statistical differences were found (Log Rank: Mantel-Cox; $\chi 2 = 25.9$; $P < 0.0001$). The baseline level of SDF observed in each sample was not determinant on the r-SDF observed in each case as the correlation coefficient obtained was very low (Pearson: r^2 0.064; $p = 0.620$).

Discussion

The results presented in this study revealed strong concordance between direct visualization of DNA damage observed after direct incorporation of labelled nucleotides using ISNT, the indirect visualization of the sperm DNA damage as detected by the N-Comet and the results obtained interpreting the haloes after using the Halomax assay. The differences in the sperm morphology observed between the N-Comet and Halomax assay are mainly due to how the DNA damage was visualized. In the Halomax assay, chromatin expansion was evidenced by detecting a peripheral halo emerging from a central core; in sperm free of DNA damage, this expansion was formed by small DNA loops. However, for sperm with significant DNA damage, the halo was substantially larger and showed a “spotty” or “stellar” configuration, which is formed by small DNA fragments emerging from a residual nucleoid core; our observations were further confirmed by the application of DNA polymerase to label the free 3´-5´ends of DNA after ISNT. The larger the amount of DNA damage, the larger the size of the haloes detected using forced-polymerase nucleotide incorporation and the smaller the size of the nuclear core. In the N-comet assay, those sperm nuclei with a “spotty” halo

were also likely to be displaced on the X-axis when the spermatozoa were subjected to an electrophoretic field. In general, DNA containing breaks is more easily affected by protein depletion since the free ends of the DNA breaks behave as origins of DNA denaturation and probably the proteins are also differentially affected after the lysing process. Basically, this is the rationale of the classical unwinding assays employed for many years for the quantification of DNA breaks in radiobiology and mutagenesis and would explain differences in the proportion of DNA damage determined when different sized haloes are compared (López-Fernández et al., 2008b; Zee et al., 2009b). Something similar is occurring when the DNA is displaced after being forced to move in an electrophoretic field when the comet assay is performed. In this case, differences in the size of the core, in the amount of DNA collected in the tail and the length of the comet displacement are all related to the differences in the amount of DNA damage per spermatozoa.

In the case of the Halomax assay and halo production, it is evident that the polymerases used in ISNT were able to incorporate labelled nucleotides into the DNA nicks inherent to the fragmented DNA with high efficiency. This nucleotide incorporation is possible because a large part of the protamines have been removed, rendering the DNA molecule more accessible to the direct action of polymerases. This is the reason why the ISNT signal is mainly concentrated at the halo, since most of the fragmented DNA is concentrated here. *In situ* polymerization of labelled nucleotides after ISNT gave rise to halos of chromatin dispersion that were larger than those detected by direct application of the fluorochrome after the Halomax assay was performed; this is probably because the size of the fragments at the most peripheral areas of the halo are formed by very small DNA fragments which do not produce DNA complexes by resonance energy transfer with the fluorochrome to produce a visible signal. The *in situ* forced expansion of the small DNA fragments after ISNT produce a more detectable signal.

The results of this investigation have shown that the level of SDF observed in rabbits is relatively low with a mean SDF value of around 9%. After removing those individuals that did not fit to a normal distribution, this value decreases to a 6.8% (SD 2.2). These SDF values are similar to those observed in other species where males were highly selected for reproductive purposes, such as Holstein bulls (González-Marín et al., 2011), boar (López-Fernández et al., 2008a) or ram (López-Fernández et al., 2008b). The presence of low values for SDF is not frequent in other species such as the human, which has a mean normal value for SDF around 15% to 25% (Gosálvez et al. 2011b; Evenson, 2013) or stallions where the threshold value for normality is on the order of a 16% (López-Fernández et al., 2007 and Gosálvez unpublished data). We suggest that a SDF around 7% can be considered as an initial estimate of sperm DNA normality for this synthetic breed until further data are obtained. Additionally, we have observed that rabbit sperm samples may exhibit a diverse range of rates of SDF; this phenomenon has been observed in all species that have been examined thus far (López-Fernández et al., 2008a;b; Johnston et al. 2012; Imrat et al., 2012) and indicated the existence of an inter-individual variation regards sperm DNA longevity. Such variation may be associated with a differential level of protamination achieved during sperm maturation and is probably dependent on genetic and "stress" related factors (Balhorn 2011; Gill-Sharma et al., 2011) during histone/protamine transition. The r-SDF associated with each sample was not dependent on the initial value of SDF observed for each sample. It would be interesting to test whether the fertilizing capacity of those sperm samples showing superior

DNA longevity (low r-SDF) was actually higher than those observed in semen samples with a high r-SDF.

While the pregnancy rate of rabbit populations in captivity is high and may range from 75% to 90 % (Castellini and Lattaioli, 1999; Viudes de Castro and Vicente, 1997), it also been shown to fluctuate with season (Tůma et al., 2010) as well as with the different use of semen extenders for artificial insemination (Carluccio et al., 2004). Improvements in reproductive efficiency in rabbits do not seem to be a priority as with other domestic animals such as bulls or stallions and this phenomenon is reflected in what appears to be the production of high quality gametes in this species, a fact which was evidenced by the relatively low level of SDF observed for most of the animals used in this experiment and the limited increase in sperm DNA fragmentation over the period of incubation.

We suggest three main reasons for assessing SDF in rabbits; (1) maintenance of sperm quality control of the hybrid lines when producing new breeds and genetic lines, (2) selection of bucks to improve reproductive potential and (3) the use of the rabbit as an experimental model to explore the relationship between sperm quality and pregnancy outcome. The rabbit is an ideal species to examine the relationship between the r-SDF and pregnancy rate, since the offspring per female is high and the turnover of female pregnancy is also high. Additionally, the r-SDF can be easily determined by means of a dynamic assessment as calculated in this experiment and the female factor could be minimized given that the same females can be inseminated using sperm with a high or a low rate of SDF.

Acknowledgments

This work was supported by Spanish Ministry of Science and Technology (MCYT: BFU2010-16738).

References

Agarwal A, Allamaneni SS. The effect of sperm DNA damage on assisted reproduction outcomes. *A review. Minerva Ginecol 2004;56:235–45.*

Balhorn R. Sperm Chromatin: an overview. In Sperm Chromatin: Biological and clinical applications in male infertility and assisted reproduction. Edited by A Zini and A Agarwal DOI 10.1007/978-1-4419-6857-9 Springer NY, Heildeberg, London. *2011.*

Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, Giwercman A. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum. Reprod. 2007;22:174-179.*

Bungum M. Sperm DNA integrity assessment: a new tool in diagnosis and treatment of fertility. *Obstet. Gynecol. Int. 2012;2012:531042 doi: 10.1155/2012/531042.*

Carluccio A, Robbe D, de Dmicis I, Contri A, Tosi U, Russo F, Paoletti M. Artificial insemination in rabbits: laboratory and field trial with three different semen extenders. *World Rabbit Sci. 2004;12:65-79.*

Castellini C, Lattaioli P. Effect of number of motile sperms inseminated on reproductive performance of rabbit does. *Anim. Reprod. Sci. 1999;57:111-120.*

Cortés-Gutiérrez E, Crespo F, Gosálvez J, Dávila-Rodriguez M, López-Fernández C, Gosálvez J. DNA fragmentation in frozen sperm of Equus asinus: Zamorano-Leonés, a breed at risk of extinction. *Theryogenology 2008; 8:1022-1032.*

Domínguez-Fandos D, Camejo MI, Ballescà JL, Oliva R. Human sperm DNA fragmentation: Correlation of TUNEL results as assessed by flow cytometry and optical microscopy. *Cytometry A 2007;71:1011-1018.*

Enciso M, López-Fernández C, Fernández JL, García P, Gosálbez A, Gosálvez J. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy *Theriogenology 2006;65: 308-16.*

Enciso M, Sarasa J, Agarwal A, Fernández JL, Gosálvez J. A two-tailed Comet assay for assessing DNA damage in spermatozoa. *RBM Online 2009;18: 609-616.*

Evenson DP. Sperm Chromatin Structure Assay (SCSA®) *Methods Mol. Biol. 2013;927:147-64.*

Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod. Tox 1991;5:115-125.*

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum. Reprod. 1999;14:1039-1049.*

Fernández JL, Vázquez-Gundín F, Rivero MT, Genescá A, Gosálvez J, Goyanes V. DBD-FISH on neutral comets: simultaneous analysis of DNA single- and double-strand breaks in individual cells. *Exp. Cell Res. 2001;270:102-109.*

Fernández JL, Vélez de la Calle JF, Tamayo M, Cajigal D, Agarwal A, Gosálvez J. Sperm DNA integrity and male infertility: current perspectives. *Arch. Med. Sci. 2009;S55-S62.*

Gill-Sharma MK, Choudhuri J, D'Souza S. Sperm chromatin protamination: an endocrine perspective. *Protein Pept. Lett. 2011; 18:786-801.*

González-Marín C, Roy R, López-Fernández C, Díez B, Carabaño MJ, Fernández ML, Kjelland ME, Moreno JF, Gosálvez J. Bacteria in bovine semen can increase sperm DNA fragmentation rates: A kinetic experimental approach. *Anim. Reprod. Sci. 2011; 123: 139–148.*

Gosálvez J, López-Fernández C, Fernández JL. Sperm Chromatin Dispersion (SCD) test: technical aspects and clinical applications. In Sperm DNA Damage: Biological and Clinical Applications in Male Infertility and Assisted Reproduction. Edit. A Zini and A Agarwal. Springer Verlag, *2011a;151-170.*

Gosálvez J, López-Fernández C, Fernández JL,Gouraud A, Holt WV. Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. *Mol. Reprod. Dev. 2011a;78:951-61.*

Gogol P, Bochenek M, Smorag Z. Sperm chromatin integrity of bucks transgenic for the WAP bGH gene. *Anim. Reprod. Sci. 2000;64:113–120.*

Gogol P, Bochenek M, Smorag Z. Effect of Rabbit Age on Sperm Chromatin Structure. *Reprod. Dom. Anim. 2002; 37:92–95.*

Imrat P, Suthanmapinanth P, Saikhun K, Mahasawangkul S, Sostaric E, Sombutputorn P, Jansittiwate S, Thongtip N, Pinyopummin A, Colenbrander B, Holt WV, Stout TA. Effect of cooled storage on quality and DNA integrity of Asian elephant (Elephas maximus) spermatozoa. *Reprod. Fertil Develop. 2012;24:1105-1116.*

Johnston SD, Satake N, Zee Y, López-Fernández C, Holt WV, Gosálvez J. Osmotic stress and cryoinjury of koala sperm: an integrative study of the plasma membrane, chromatin stability and mitochondrial function. *Reproduction 2012; 143:787-97.*

Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril 1998;69:528–532.*

López-Fernández C, Pérez-Llano B, García-Casado P, Sala R, Gosálbez A, Arroyo F, Fernández JL, Gosálvez J. Sperm DNA *fragmentation* in a random sample of the Spanish boar livestock. *Anim. Reprod. Sci. 2008a;103:87-98.*

López-Fernández C, Fernández JL, Gosálbez A, Arroyo F, Vázquez JM, Gosálvez J. Dynamics of sperm DNA fragmentation in domestic animals III. Ram *Therigenology 2008 b;70: 898-908.*

López-Fernández C, Crespo F, Arroyo F, Fernández JL, Arana P, Johnston SD, Gosálvez J. Dynamics of sperm DNA fragmentation in domestic animals II. The stallion. *Theriogenology 2007; 68:1240-1250.*

Portas T, Johnston SD, Hermes R, Arroyo F, López-Fernández C, Bryant B, Hildebrandt T, Göritz F, Gosálvez J. Frozen-thawed rhinoceros sperm exhibit DNA damage shortly after thawing when assessed by the sperm chromatin dispersion assay. *Theriogenology 2009;72:711-720*.

Tůma J, Tůmová E, Valášek V. The effect of season and parity order on fertility of rabbit does and kit growth. *Czech J. Anim. Sci. 2010; 55:330–336.*

Viudes De Castro MP, Vicente JS. Effect of sperm count on the fertility and prolificity rates of meat rabbits. *Anim. Reprod. Sci. 1997;46:313-319.*

Zini A, Libman J. Sperm DNA damage: clinical significance in the era of assisted reproduction. *CMAJ. 2006;175:495-500.*

Zee YP, López-Fernández C, Gosálvez J, HolT WV, Johnston SD. DNA fragmentation dynamics of koala spermatozoa. *Reprod. Fertil Develop 2009a; 21:141-142.*

Zee YP, López-Fernández C, Arroyo F, Johnston SD, Holt WV and Gosálvez J. Evidence that single-stranded DNA breaks are a normal feature of koala sperm chromatin, while double-stranded DNA breaks are indicative of DNA damage *Reproduction 2009b;138: 267–278.*

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter VI

Nutritional Effects of Plant Oil and Seeds in Rabbit Feeding

P. G. Peiretti [*] ***and F. Gai***
Institute of Sciences of Food Production, Italian
National Research Council, Grugliasco, Italy

Abstract

This study aims to review various rabbit experiments on the effects of supplementating mixed feed with plant oils (corn and palm) or oilseeds (false flax, golden flax, chia, and perilla seeds). Apparent digestibility and lipid traits in the *longissimus dorsi* muscle of growing rabbits fed with these fat sources are reviewed. Moreover, our results are compared with similar works on rabbit nutrition.

Fat sources influence the fatty acid (FA) composition of rabbit body fat. Even though meat and meat products are often associated with nutrients considered to be unhealthy, in fact rabbit meat offers excellent nutritive and dietetic properties. Further improvements to rabbit meat production can be achieved by adding functional compounds associated with animal feed fortification or enrichment. Among these functional compounds long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) have many known beneficial effects on health.

False flax, golden flax and chia seed supplementation in mixed feed increases the apparent digestibility of most of the nutrients, and false flax and golden flax in particular improved ether extract digestibility in weaned crossbred rabbits. A similar result was also obtained in rabbits fed mixed feed supplemented with corn oil, with less impressive resultsfor those supplemented with palm oil.

The FA profile in rabbit meat mainly reflects the dietary oil sources. The saturated FA and monounsaturated FA contents were lower in the muscles of animals fed the corn oil diet than in those of rabbits given the palm oil diet. In contrast, total PUFA and n-6 PUFA content was lower in the muscles of rabbits fed the unsaturated palm oil diet than in animals given the corn oil diet.

[*] Corresponding author: P. G. Peiretti. Tel.: +39 11 6709230; fax: +39 11 6709297; E-mail address: piergiorgio.peiretti@ispa.cnr.it.

In rabbits fed a diet with increasing quantities of oilseed, the saturated FA and monounsaturated FA proportions in the *longissimus dorsi* muscle decreased, while PUFA increased. Oilseed dietary supplementation has shown to be effective in raising the n-3 PUFA proportion in the meat and decreasing the n-6/n-3 PUFA ratio.

In conclusion, these results showed that the use of diets with corn oil or supplemented with different oilseeds was effective in reducing the saturation, atherogenic and thrombogenic indexes, with consequent benefits on the nutritional quality of rabbit meat for consumers, with no significant adverse effects on digestibility in growing rabbits.

Keywords: Rabbit, digestibility, meat quality, fatty acid, oilseed

Introduction

One of the main aims of rabbit meat researchers is to increase the unsaturated fatty acids (FA) content and reduce the saturated FAs in fat deposits, in order to produce healthy and nutritious meat (Dalle Zotte, 2002). Rabbit meat offers excellent nutritive properties (Dalle Zotte, 2004; Combes, 2004; Combes and Dalle Zotte, 2005; Hernández and Gondret, 2006). It is highly valued because of its dietary properties, since it is a lean meat with good nutritional value, low-fat content and lower quantities of saturated FAs and cholesterol than other meats (Hernández, 2008). The FA profile of rabbit meat will have been influenced by the composition of the animal's diet, its digestive system and its biosynthetic processes (Woods and Fearon, 2009); in fact, the fat content and FA profile of rabbit meat depend on the feeding, age, genotype, breeding and/or physical activity of the animals as well as on the sex of the animals and the muscle type in question (Ramírez et al., 2005; Polak et al., 2006).

Ingredients commonly used in rabbit diets contain low level of lipids (< 2.5%); therefore, in order to improve the energy and lipid quality content of the diet, some vegetable oils or whole oilseeds could be added to the mixed feed with the aim of improving its digestibility and the meat FA profile (Maertens, 1998).

Rabbit meat could play a valuable role in human nutrition due to all these characteristics and the possibility of manipulating the composition of the FAs through diet (Oliver et al., 1997). Indeed, the rabbit meat FA profile can be modified through the strategic use of unsaturated dietary fat sources (Dalle Zotte, 2002), because rabbits, like other monogastric animals, are able to directly incorporate dietary FAs into adipose and intramuscular tissue lipids. Therefore, feeding has the highest impact on meat quality and many studies have reported that the FA profile of rabbit muscles can effectively be modified when rabbits are fed with diets with different FA supplementations even after only a month of treatment (Hernández et al., 2000; Szabó et al., 2003).

The content of polyunsaturated FA (PUFA) in rabbit meat is relatively high (Ouhayoun et al., 1987; Alasnier and Gandemer, 1998), but a great deal of research has focused on increasing n-3 PUFA content and lowering the n-6/n-3 PUFA ratio in rabbit meat through diet. As for other monogastric animals, this modification may be performed by supplementing diets with vegetable oil or raw materials rich in n-3 PUFA content (Dalle Zotte and Szendrő, 2011). Morever Peiretti (2012) studied the kinetics and quantitative relationship of FA deposits in rabbit meat with the aim of modelling the relationships between the FA profile in rabbit tissues and feeds.

In order to compare the effects of different vegetable oils or different inclusion levels of non-conventional seeds on rabbit nutrient utilisation, different nutritional experiments were carried out using diets for crossbred rabbits supplemented with palm and corn oil, false flax (*Camelina sativa* L.), golden flax (*Linum usitatissimum* L.), perilla (*Perilla frutescens* L.) and chia (*Salvia hispanica* L.), respectively. The apparent digestibility coefficient of dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF) and gross energy (GE) of these diets, were investigated in the works of Zunino et al. (2010), Peiretti et al. (2007a), Peiretti and Meineri (2008a), Peiretti et al. (2010) and Meineri and Peiretti (2007).

This review also examines the works of Peiretti et al. (2007b, 2011a, 2011b), Peiretti and Meineri (2008b, 2010) on the effects of different vegetable oils or different inclusion levels of non-conventional seeds as possible sources of unsaturated FAs for inclusion in rabbit diets and their subsequent transfer into meat in order to improve its quality.

Oilseed and Plant Oil Chemical Composition

Table 1 reports the FA profile of diets supplemented with palm oil (PO) and corn oil (CO), respectively. The most abundant FAs in the PO diet were oleic acid (OA, C18:1n-9) and palmitic acid (PA, C16:0), whereas the CO diet was rich in linoleic acid (LA, C18:2n-6). The FA pattern of the diets was characterised by higher percentages of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) and lower percentages of PUFA in the PO diet than in the CO diet. Moreover, due to the high LA content, the n-6/n-3 ratio in the CO diet was higher than in the PO diet.

Table 1. Fatty acids composition (g/100 g total fatty acids) of diets supplemented with different oils

	Palm oil	Corn oil
Myristic acid	6.1	0.0
Palmitic acid	297.4	168.2
Palmitoleic acid	3.5	0.0
Stearic acid	59.0	27.1
Oleic acid	343.3	279.0
Vaccenic acid	9.1	6.4
Linoleic acid	233.2	461.2
Linolenic acid	40.7	42.2
Arachidonic acid	0.0	4.1
Other	7.8	11.8
n-6/n-3	5.73	11.0
S/P	0.57	0.24

Adapted from Peiretti et al., 2011a. n-6/n-3: polyunsaturated fatty acid series n-6/polyunsaturated fatty acid series n-3 ratio. S/P: saturated fatty acid/unsaturated fatty acid ratio.

The chemical composition of the oilseeds in the study (false flax, golden flax, perilla and chia seeds) is reported in Table 2. DM and CP ranged between 93.2 and 95.3% and between 23.5 and 24.5%, respectively. Perilla seed showed the highest EE content (43.0%) while chia seed showed the highest percentages of LA and linolenic acid (ALA, C18:3n-3).

Mixed Feed Digestibility

Rabbits have proved to be able to utilise various conventional oleaginous seeds, such as cotton, flaxseed, soybean, safflower (Johnston and Berrio, 1985) and sunflower (Balogun and Etukude, 1991). Zunino et al. (2010) evaluated the effect of the inclusion of plant oils in rabbit diets on the apparent digestibility of mixed feeds; these authors observed that the percent apparent digestibility coefficients of CP were higher in the PO supplemented diet than in the CO diet (Table 3).

By contrast, CO supplementation resulted in better EE digestibility than the PO supplemented diet. Similar values of EE digestibility for full-fat soybeans or rapeseed were found by Maertens et al. (1996). This could be due to the fact that the digestibility of the unsaturated fat fraction is higher than the saturated fat fraction, which in turn shows that the EE digestibility of diets containing CO (rich in unsaturated FAs) is higher than that of diets with PO (rich in saturated FAs).

Similar results were found by Fernández et al. (1994), who demonstrated that EE digestibility improves in diets with oil containing unsaturated FA (soyabean oil) compared to diets with saturated fats of animal origin (beef tallow).

Table 2. Chemical composition (%), gross energy (MJ/kg DM) and main fatty acids (g/100 g total fatty acids) of the seeds used to supplement experimental diets

	False flax	Golden flax	Perilla	Chia
DM	93.2	93.3	95.3	94.9
OM	96.8	96.6	96.2	95.2
CP	24.5	23.5	23.9	23.5
CF	33.3	17.1	22.6	32.9
EE	30.2	36.9	43.0	31.1
Ash	3.2	3.4	3.8	4.8
NFE	8.7	19.1	6.8	7.8
GE	28.1	27.0	28.0	26.1
Linoleic acid	17.7	15.9	16.2	18.8
Linolenic acid	37.3	58.3	62.0	64.1

Adapted from Peiretti et al., 2007b; Peiretti and Meineri, 2010; Peiretti et al., 2011b; Peiretti and Meineri, 2008b.

DM: dry matter, OM: organic matter, CP: crude protein, CF: crude fibre, EE: ether extract, NFE: Nitrogen free extract, GE: gross energy.

Table 3. Percent apparent digestibility coefficients, obtained using AIA (acid insoluble ash) as the internal marker

	Palm oil	Corn oil
DM	65.5	66.3
OM	65.4	65.9
CP	72.3	69.9
EE	83.0	88.3
NDF	33.8	34.2
ADF	25.2	24.2
GE	66.3	66.6

Adapted from Zunino et al., 2010.

DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fibre, ADF: acid detergent fibre, GE: gross energy.

Table 4. Percent apparent digestibility coefficients, obtained using acid insoluble ash as the internal marker

	False flax			Golden flax			Perilla			Chia		
	0%	10%	15%	0%	8%	16%	0%	5%	10%	0%	10%	15%
DM	63.2	68.0	68.3	65.4	63.2	67.7	67.2	65.2	66.6	68.7	72.4	72.2
OM	64.9	69.9	70.7	66.2	63.9	68.5	67.3	65.2	66.5	69.1	73.4	73.0
CP	68.5	69.5	66.2	65.0	65.7	68.4	70.2	68.8	69.2	71.2	72.7	73.6
EE	85.2	91.9	93.0	83.5	87.3	90.6	73.8	77.5	83.9	89.1	91.8	85.2
NDF	29.1	25.2	25.8	27.0	26.9	32.5	27.7	23.2	25.2	32.2	30.4	28.8
ADF	30.7	28.3	26.1	25.3	20.1	31.9	21.8	18.6	20.9	23.3	24.5	19.0
GE	64.3	69.7	70.7	65.2	64.5	69.2	66.4	64.2	66.5	65.5	69.4	69.7

Adapted from Peiretti et al., 2007a; Peiretti and Meineri, 2008a; Peiretti et al., 2010; Meineri and Peiretti 2007.

DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fibre, ADF: acid detergent fibre, GE: gross energy.

Table 4 reports the apparent digestibility coefficients of DM, OM, CP, EE, NDF, ADF and GE of diets supplemented with four non-conventional seeds (false flax, golden flax, perilla and chia) derived from the data of Peiretti et al. (2007a), Peiretti and Meineri (2008a), Peiretti et al. (2010) and Meineri and Peiretti (2007), respectively.

Mixed feed with the highest inclusion levels of false flax (15%), golden flax (16%) and chia (15%) seeds showed an improved digestibility of DM, OM and GE. Maximum values of apparent digestibility coefficients for DM (72.2%) and OM (73.0%) were obtained using the chia diet, while the false flax diet showed the maximum value of GE digestibility (70.7%).

As far as EE digestibility is concerned, it increased with increasing inclusion levels of false flax (15%), golden flax (16%) and perilla (10%) seeds with the maximum digestibility value (93%) being recorded in animals fed a diet supplemented with 15% false flax. Similar values have been reported for whole soybeans and for colza seeds (Maertens and De Groote,1984; Maertens et al., 1996).

EE digestibility is generally higher when the level of dietary fat is increased and its value usually depends on the type of added fat (Pascual et al., 2002). Van Manen et al. (1989) described an increase in EE digestibility when fat was added to the diet and this could be due to the fact that with increasing fat intake the faecal excretion of endogenous fat had a diminishing effect on the calculated apparent digestibility.

As regards digestibility of the fibre fractions (NDF and ADF), higher inclusion levels of false flax (15%), perilla (10%) and chia (15%) seeds decreased the digestibility values of both fibre fractions. The lowest NDF and ADF values were recorded in animals fed with perilla (10%) and chia (15%) supplemented with diets containing values of 25.2 and 19.0%, respectively. Contrasyingly, the group fed with golden flax (16%) seeds reported 32.5 and 31.9% digestibility values for NDF and ADF, respectively, showing an opposite trend to other seeds. A similar result was found by Fernández et al. (1994), who observed an increase in ADF digestibility when supplemented fat was added to the diet, but in most of the works, authors found no significant differences in the digestibility of fibre fractions (Xiccato et al., 1995; Pérez et al., 1996). These authors stated that the differences seem to be attributed more to changes in dietary fibre content and nature than to the addition of fat itself.

Meat Quality

As regards FA composition of the meat, the FA profile and indexes related to human health in the *longissimus dorsi* muscle of rabbits fed diets supplemented with PO or CO is reported in Table 5. The muscle of rabbits fed the more saturated PO diet presented higher SFA and MUFA contents than those of the rabbits fed a CO diet. By contrast, total PUFA content was lower in muscles of rabbits fed the unsaturated PO diet (23.9%) than of animals fed a CO diet (34.5%). Dalle Zotte and Szendrő (2011) reported in a recent review, that unsaturated FAs and PUFA amount represent around 60% and 32.5% of total FAs in rabbit meat, repectively. In order to increase the level of lipid unsaturation, different vegetable oil sources have already been used in rabbit diets (Dalle Zotte, 2002). Oils derived from sunflower, soybean or rapeseed supplemented in the rabbit diet, produced a highly unsaturated meat, consisting mainly in α-linolenic acid and linoleic acid (Cobos et al., 1993; Cavani et al., 1996). Hernández et al. (2007) found higher percentages of linolenic and linoleic acid in the leg meat of rabbits fed with the diet enriched with 3% flaxseed oil and 3% sunflower oil, respectively, than in the animals fed with the diet enriched with 3% animal fat. Among vegetable oils, flaxseed oil was the most common source of α-linolenic acid as a way of raising n-3 PUFA content and reducing the n-6/n-3 PUFA ratio in rabbit meats (Bernardini et al., 1999; Dal Bosco et al., 2004; Colin et al., 2005; Hernández et al., 2007). Comparing the effect of dietary fat supplementation with sunflower, palm and coconut oils, Gondret et al. (1998) showed that the lipids of meat fat contained significantly more SFA when rabbits were fed with coconut oil, while PUFA content was highest with the sunflower oil.

Contrastingly, Lopez-Bote et al. (1997) showed that the supplementation of sunflower oil or olive oil reduced n-3 PUFA content in polar lipids, compared with rabbits fed unsupplemented diets.

CO supplementation improved all the rabbit meat indexes related to human health compared to PO supplementation, but it negatively affected the n-6/n-3 PUFA ratio that increased. The S/P ratio was lower in the muscle of rabbits fed with the CO supplemented diet than with the PO diet (0.55 *vs* 0.67).

This result is in agreement with Gondret et al. (1998), who found a decreasing S/P ratio in the tissues of growing rabbits fed diets with coconut oil (from 1.1 to 1.6), palm oil (0.7) or sunflower oil (from 0.5 to 0.6).

Generally, the dietary enrichment with vegetable oils rich in α-linolenic acid increase unsaturation of depot lipids in rabbit fat (Oliver et al., 1997) and reduce their n-6/n-3 PUFA ratio (Dal Bosco and Castellini, 1998).

Table 6 summarises the FA composition and indexes related to human health in the *longissimus dorsi* muscle of rabbits fed with different levels of four non-conventional seeds. Mixed feeds with higher inclusion levels of these oilseeds led to decreasing SFA content in meat, while PUFA content increased with increasing supplementation levels. This increase is due to the abundance of α-linolenic acid in oilseed, which helps to improve n-3 PUFA content and to lower the n-6/n-3 PUFA ratio in rabbit meat. The saturation, atherogenic and thrombogenic indexes were significantly lower in rabbits fed the oilseed supplemented diet compared to rabbits fed the control diet.

Concerning oilseed utilisation as raw material in rabbit feed, more recently the use of flaxseed was tested.

Table 5. Fatty acid composition (g/100 g total fatty acids) and indexes related to human health in the *longissimus dorsi* muscle of rabbits

	Palm oil	Corn oil
SFA	39.4	34.6
MUFA	34.9	29.2
PUFA	23.9	34.5
n-3 PUFA	2.3	2.5
n-6 PUFA	21.5	32.0
n-6/n-3	9.55	13.09
S/P	0.67	0.55
AI	0.69	0.55
TI	1.12	0.91

Adapted from Peiretti et al., 2011a.

SFA: Saturated Fatty Acid, MUFA: Monounsaturated Fatty Acid, PUFA: Polyunsaturated Fatty Acid, n-3 PUFA: Polyunsaturated Fatty Acid series n-3, n-6 PUFA: Polyunsaturated Fatty Acid series n-6, n-6/n-3: n-6 PUFA/n-3 PUFA ratio, S/P: Saturated Fatty Acid/Unsaturated Fatty Acid, AI: Atherogenic Index, TI: Thrombogenic Index.

Table 6. Fatty acid composition (g/100 g total fatty acids) and indexes related to human health in the *longissimus dorsi* muscle of rabbits

	False flax			Golden flax			Perilla			Chia		
	0%	10%	15%	0%	8%	16%	0%	5%	10%	0%	10%	15%
SFA	37.9	30.8	29.0	34.9	31.8	27.3	40.8	35.7	32.9	39.7	30.6	27.9
MUFA	30.8	30.2	30.8	32.1	27.8	24.5	35.7	29.3	27.4	30.2	20.2	16.8
PUFA	30.2	38.0	39.6	27.2	35.1	42.7	22.9	34.1	39.1	27.7	45.6	50.7
n-3 PUFA	6.2	14.9	18.1	4.9	12.6	20.3	3.1	14.7	19.7	5.0	20.9	25.2
n-6 PUFA	23.9	23.1	21.5	22.3	22.5	22.5	19.8	19.4	19.5	22.7	24.7	25.6
n-6/n-3	3.86	1.57	1.19	4.58	1.85	1.13	6.53	1.35	1.00	4.55	1.19	1.03
S/P	0.60	0.44	0.40	0.58	0.49	0.39	0.68	0.56	0.48	0.67	0.45	0.40
AI	0.64	0.47	0.43	0.59	0.49	0.38	0.66	0.54	0.47	0.68	0.44	0.37
TI	0.79	0.42	0.35	0.81	0.49	0.31	1.08	0.51	0.39	0.93	0.35	0.28

Adapted from Peiretti et al., 2007b; Peiretti and Meineri, 2010; Peiretti et al., 2011b; Peiretti and Meineri, 2008b.

SFA: Saturated Fatty Acid, MUFA: Monounsaturated Fatty Acid, PUFA: Polyunsaturated Fatty Acid, n-3 PUFA: Polyunsaturated Fatty Acid series n-3, n-6 PUFA: Polyunsaturated Fatty Acid series n-6, n-6/n-3: n-6 PUFA/n-3 PUFA ratio, S/P: Saturated Fatty Acid/Unsaturated Fatty Acid, AI: Atherogenic Index, TI: Thrombogenic Index.

Bianchi et al. (2006) showed a significant decrease in the n-6/n-3 PUFA ratio when the effect of dietary use of whole flaxseed (8%) on rabbit meat quality was studied. In a study on the influence of the dietary use of whole flaxseed (3, 6 or 9%) on rabbit meat quality, Bianchi et al. (2009) found that the PUFA n-3 content of *longissimus dorsi* muscle increased significantly with the increasing level of flaxseed in the diet, mainly due to the higher content of α-linolenic acid, which also led to a reduction in the n-6/n-3 PUFA ratio while SFA content fell significantly.

Ander et al. (2010) found that dietary flaxseed is an effective supplement for lowering the n-6/n-3 PUFA ratio in numerous pathologically relevant tissues in the rabbit and produced a significant increase in α-linolenic acid, with a preferential distribution to the heart and liver. They concluded that flaxseed may be a useful dietary supplement for any condition in which an elevation of n-3 PUFA would be expected to produce positive health effects. Recently, the use of whole white lupin seeds as a novel FA source to increase levels of beneficial MUFA and PUFA in rabbit meat was investigated by Volek and Marounek (2011).

They found that diets supplemented with white lupin seeds fed to rabbits significantly decreased SFA and PUFA content, as well as the n-6/n-3 PUFA ratio and saturation, atherogenic and thrombogenic indexes in hind leg meat when compared with rabbit fed diets supplemented with sunflower meal.

Conclusion

Our research has shown that these non-conventional seeds may be used satisfactorily as a nutrient supplement for rabbits in their diet, without any adverse effects on growth performance and with better digestibility than in the control diet. Moreover the supplementation with these oilseeds is effective in improving n-3 PUFA content, decreasing the n-6/n-3 PUFA ratio and reducing the saturation, atherogenic and thrombogenic indexes of the meat and fat, with consequent benefits for consumers interm of the nutritional quality of rabbit meat.

References

Alasnier, C. and Gandemer, G. (1998). Fatty acid and aldehyde composition of individual phospholipid classes of rabbit skeletal muscles is related to the metabolic type of the fibre. *Meat Science*, 48, 225-235.

Ander, B. P., Edel, A. L., McCullough, R., Rodriguez-Leyva, D. R., Rampersad, P., Gilchrist, J. S. C., Lukas, A., and Pierce, G. N. (2010). Distribution of omega-3 fatty acids in tissues of rabbits fed a flaxseed supplemented diet. *Metabolism, Clinical and Experimental*, 59, 620-627.

Balogun, T. F. and Etukude, U. W. (1991). Undecorticated full-fat sunflower seeds in the diet of rabbits. *Journal of Applied Rabbit Research*, 14, 101-104.

Bernardini, M., Dal Bosco, A. and Castellini, C. (1999). Effect of dietary n-3/n-6 ratio on fatty acid composition of liver, meat and perirenal fat in rabbits. *Animal Science*, 68, 647-654.

Bianchi, M., Petracci, M. and Cavani, C. (2006). Effects of dietary inclusion of dehydrated lucerne and whole linseed on rabbit meat quality. *World Rabbit Science*, 14, 247-258.

Bianchi, M., Petracci, M., Cavani, C. (2009). The influence of linseed on rabbit meat quality. *World Rabbit Science*, 17, 97-107.

Cavani, C., Zucchi, P., Minelli, G., Tolomelli, B., Cabrini, L., and Bergami, R. (1996). Effect of soybeans on growth performance and body fat composition in rabbits. In: *Proceedings of the 6th World Rabbit Congress*, Toulouse, France, pp. 127-133.

Cobos, A., Cambero, M. I., Ordotiez, J. A., and de la Hoz, L. (1993). Effect of fat enriched diets on rabbit meat fatty acid composition. *Journal of the Science of Food and Agriculture*, 62, 83-88.

Colin, M., Raguanes, N., Le Berre, G., Charrier, S., Prigent, A. Y., and Perrin, G. (2005). Influence d'un enrichissement de l'aliment en acides gras oméga 3 provenant de graines de lin extrudées (Tradi-Lin®) sur le lipids et les caractéristiques hédoniques de la viande de lapin. In: *Proceeding of the 11èmes Journées de la Recherche Cunicole*, Paris, France, pp.163-166.

Combes, S. (2004). Valeur nutritionnelle de la viande de lapin. *INRA Productions animales*, 17, 373-383.

Combes, S. and Dalle Zotte, A. (2005). La viande de lapin: valeur nutritionnelle et particularités technologiques. In: *Proceeding of the 11èmes Journées de la Recherche Cunicole*, Paris, France, pp. 167-180.

Dal Bosco, A. and Castellini, C. (1998). Effets de l'addition de vitamine E dans l'aliment et des conditions de conservation des carcasses sur les caractéristiques physico-chimiques de la viande chez le lapin. In: *Proceeding of the 7èmes Journées de la Recherche Cunicole*, Lyon, France, pp. 111-114.

Dal Bosco, A., Castellini, C., Bianchi, L., and Mugnai, C. (2004). Effect of dietary α-linolenic acid and vitamin E on the fatty acid composition, storage stability and sensory traits of rabbit meat. *Meat Science*, 66, 407-413.

Dalle Zotte, A. (2002). Perception of rabbit meat quality and major factors influencing the rabbit carcass and meat quality. *Livestock Production Science*, 75, 11-32.

Dalle Zotte, A. (2004). Avantages diététiques. Le lapin doit apprivoiser le consommateur. *Viandes Produits Carnés*, 23, 1-7.

Dalle Zotte, A. and Szendrő, Z. (2011). The role of rabbit meat as functional food. *Meat Science*, 88, 319-331.

Fernández, C., Cobos, A. and Fraga, M. J. (1994). The effect of fat inclusion on diet digestibility in growing rabbits. *Journal of Animal Science*, 72, 1508-1515.

Gondret, F., Mourot, J., Lebas, F., and Bonneau, M. (1998). Effects of dietary fatty acids on lipogenesis and lipid traits in muscle, adipose tissue and liver of growing rabbits. *Animal Science*, 66, 483–489.

Hernández, P., Pla, M., Oliver, M. A., and Blasco, A. (2000). Relationships between meat quality measurements in rabbits fed with three diets with different fat type and content. *Meat Science*, 55, 379-384.

Hernández, P. and Gondret, F. (2006). Rabbit meat quality. In: Maertens, L. and Coudert, P. (Eds.), *Recent advances in rabbit sciences* (pp. 269-290). Melle, Belgium: ILVO.

Hernández, P., Cesari, V. and Pla, M. (2007). Effect of the dietary fat on fatty acid composition and oxidative stability of rabbit meat. In: *Proceeding of the 53rd International Congress of Meat Science and Technology*, Beijing, China, pp. 367-370.

Hernández, P. (2008). Enhancement of nutritional quality and safety in rabbit meat. In: *Proceeding of the 9th World Rabbit Congress*, Verona, Italy, pp. 1287-1299.

Johnston, N. P. and Berrio, L. F. (1985). Comparative effects of cottonseed, soybeans, safflower seeds and flax seeds on the performance of rabbits and guinea pigs. *Journal of Applied Rabbit Research*, 8, 64-67.

Lopez-Bote, C., Rey, A., Ruiz, J., Isabel, B., and Sanz Arias, R. (1997). Effect of feeding diets high in monounsaturated fatty acids and α-tocopheryl acetate to rabbits on resulting carcass fatty acid profile and lipid oxidation. *Animal Science*, 64, 177-186.

Maertens, L. and De Groote, G. (1984). Digestibility and digestible energy content of a number of feedstuffs for rabbits. In: *Proceedings of the 3rd World Rabbit Congress*, Rome, Italy, pp. 244-251.

Maertens, L., Luzi, F. and Huybrechts, I. (1996). Digestibility of nontransgenic and transgenic oilseed rape in rabbits. In: *Proceedings of the 6th World Rabbit Congress*, Toulouse, France, pp. 231-236.

Maertens, L. (1998). Fats in rabbit nutrition: a review. *World Rabbit Science*, 6, 341-348.

Meineri, G. and Peiretti P. G. (2007). Apparent digestibility of mixed feed with increasing levels of chia (*Salvia hispanica* L.) seeds in rabbit diets. *Italian Journal of Animal Science*, 6, 778-780.

Oliver, M. A., Guerrero, L., Diaz, I., Gispert, M., Pla, M., and Blasco, A. (1997). The effect of fat-enriched diets on the perirenal fat quality and sensory characteristics of meat from rabbits. *Meat Science*, 47, 95-103.

Ouhayoun, J., Kopp, J., Bonnet, M., Demarne, Y., and Delmas, D. (1987). Influence de la composition des graisses alimentaires sur les propriétés des lipides périrenéaux et la qualité de la viande de lapin. *Sciences des Aliments*, 7, 521-534.

Pascual, J. J., Motta, W., Cervera, C., Quevedo, F., Blas, E., and Fernández-Carmona, J. (2002). Effect of dietary energy source on the performance and perirenal fat thickness evolution of primiparous rabbit does. *Animal Science*, 75, 267-279.

Peiretti, P. G., Mussa, P. P., Meineri, G., and Perona, G. (2007a). Apparent digestibility of mixed feed with increasing levels of false flax (*Camelina sativa* L.) seeds in rabbit diets. *Journal of Food, Agriculture and Environment*, 5, 85-88.

Peiretti, P. G., Mussa, P. P., Prola, L., and Meineri, G. (2007b). Use of different levels of false flax (*Camelina sativa* L.) seed in diets for fattening rabbits. *Livestock Science*, 107, 192-198.

Peiretti, P. G. and Meineri, G. (2008a). Effects of golden flaxseed supplementation on the performance and feed digestibility of rabbits. *Journal of Animal and Veterinary Advances*, 7, 56-60.

Peiretti, P. G. and Meineri, G. (2008b). Effects on growth performance, carcass characteristics, and the fat and meat fatty acid profile of rabbits fed diets with chia (*Salvia hispanica* L.) seed supplements. *Meat Science*, 80, 1116-1121.

Peiretti, P. G., Gai, F., Meineri, G., Zoccarato, I., and Gasco, L. (2010). Apparent digestibility of compound diets with increasing levels of perilla (*Perilla frutescens* L.) seeds in rabbits. *Italian Journal of Animal Science*, 9, 425-428.

Peiretti, P. G. and Meineri, G. (2010). Effects of diets with increasing levels of golden flaxseed on carcass characteristics, meat quality and lipid traits of growing rabbits. *Italian Journal of Animal Science*, 9, 372-377.

Peiretti, P. G., Masoero, G. and Meineri, G. (2011a). Effects of replacing palm oil with maize oil and *Curcuma longa* supplementation on the performance, carcass characteristics, meat quality, and fatty acid profile of the perirenal fat and muscle of growing rabbits. *Animal*, 5, 795-801.

Peiretti, P. G., Gasco, L., Brugiapaglia, A., and Gai, F. (2011b). Effects of perilla (*Perilla frutescens* L.) seeds supplementation on performance, carcass characteristics, meat quality and fatty acid composition of rabbits. *Livestock Science*, 138, 118-124.

Peiretti, P. G. (2012). Effects of dietary fatty acids on lipid traits in the muscle and perirenal fat of growing rabbits fed mixed diets. *Animals*, 2, 55-67.

Pérez, J. M., Fortun-Lamothe, L. and Lebas, F. (1996). Comparative digestibility of nutrients in growing rabbits and breeding does. In: *Proceedings of the 6th World Rabbit Congress*, Toulouse, France, pp. 267-270.

Polak, T., Gašperlin, L., Rajar, A., Žlender, B. (2006). Influence of genotype lines, age at slaughter and sexes on the composition of rabbit meat. *Food Technology and Biotechnology*, 44, 65-73.

Ramírez, J. A., Díaz, I., Pla, M., Gil, M., Blasco, A., and Oliver, M. A. (2005). Fatty acid composition of leg meat and perirenal fat of rabbits selected by growth rate. *Food Chemistry*, 90, 251-256.

Szabó, A., Husvéth, F., Szendrő, Z., Repa, I., and Romvári, R. (2003). Effects of transcutaneous electrical nerve stimulation on the fatty acid profile of rabbit *longissimus dorsi* muscle (preliminary report). *Journal of Animal Physiology and Animal Nutrition*, 87, 309-314.

Van Manen, D. G., Verstegen, M. W. A., Meijer, G. W., and Beynen, A. C. (1989). Growth performance by rabbits after isoenergetics substitution of dietary fat for carbohydrates. *Nutritional Reproduction International*, 40, 443-450.

Volek, Z. and Marounek, M. (2011). Effect of feeding growing–fattening rabbits a diet supplemented with whole white lupin (*Lupinus albus* cv. Amiga) seeds on fatty acid composition and indexes related to human health in hind leg meat and perirenal fat. *Meat Science*, 87, 40-45.

Woods, V. B. and Fearon, A. M. (2009). Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: a review. *Livestock Science*, 126, 1-20.

Xiccato, G., Parigi-Bini, R., Dalle Zotte, A., Carazzolo, A., and Cossu, M. E. (1995). Effect of dietary energy level, addition of fat and physiological state on performance and energy balance of lactating and pregnant rabbit does. *Animal Science*, 61, 387-398.

Zunino, V., Meineri, G. and Peiretti, P. G. (2010). *Curcuma longa* and dietary plant oils for growing rabbits. Effects on apparent digestibility. *Journal of Food, Agriculture and Environment*, 8, 435-438.

In: Rabbits: Biology, Diet and Eating Habits and Disorders ISBN: 978-1-62948-267-5
Editors: Gianni Adamo and Albert Costanza

Chapter VII

Current Studies on the Etiology of Obstructive Dysfunction of the Male Rabbit Urinary Bladder

Connor M. Callaghan, Catherine Schuler, Robert E. Leggett and Robert M. Levin*
Albany College of Pharmacy and Health Sciences
Stratton VA Medical Center, Albany, NY, US

Abstract

Bladder dysfunction secondary to benign prostatic hyperplasia (BPH) is a major affliction of aging men. Although the symptoms of BPH are related to the effects of an enlarging prostate there appears to be no direct relationship between prostate size and severity of obstructive bladder dysfunction. Therefore, urodynamic findings cannot accurately predict either level of bladder pathology or potential for recovery following surgery or pharmacological therapy. Thus, biomarkers that can identify the severity of male obstructive bladder dysfunction and at what point the dysfunction becomes irreversible would be of significant value in the management of the disorder.

The progression of obstructive bladder dysfunction from compensated bladder function through severe bladder decompensation is mediated primarily by four processes: **1)** Cyclical ischemia followed by reperfusion (I/R) mediated by the hypertrophied bladder smooth muscle and compression of the blood vessels during contraction. **2)** Increase in intracellular free calcium mediated by the ischemia resulting in activation of specific intracellular proteases, lipases, and phospholipases that result in cellular and intracellular damage. **3)** The progressive increase in the severity of the I/R results in increased free radical generation that cannot be handled by the intracellular antioxidant mechanisms such as superoxide dismutase (SOD) and catalase resulting in progressive obstructive bladder dysfunction. And **4)** progressive replacement of functional smooth

* Contact Information: Robert M. Levin PhD, Senior Research Career Scientist, Stratton VA Medical Center, Holland Ave., Albany, NY 12208. 518-626-5662; Robert.levin2@va.gov.

muscle with connective tissue mediated by the cellular and subcellular damage to the muscle resulting in irreversible full stage obstructive dysfunction.

Introduction

The function of the urinary bladder is to collect and store urine at low intravesical pressure and then periodically expel the urine via a highly coordinated and sustained contraction [1]. Its function is no less important than the beating of the heart or breathing mediated by the lungs. Unfortunately, it is often overlooked in terms of research for a few reasons. The prevalence of death due to disorders of the bladder is much less than other organs such as the heart or lungs. However, all play an integral role in the function of the body, an incredibly intricate balance of different organ systems which is so efficient we often take it for granted until something goes wrong. As such, our duty as researchers compels us to understand both the everyday function and the potential dysfunctions that may arise in order to mitigate further complications and allow people to enjoy their lives. This begins by reaching a thorough and comprehensive understanding of the prostate, bladder, and urethra when discussing lower urinary tract problems of men.

Obstructive bladder dysfunction in men is the most common dysfunction. Its incidence and severity is related to age and although generally not life threatening, it can lead to significant dysfunctions and can interfere with normal everyday life. In general, as men age the prostate enlarges and by the age of about 40 it can begin to compress the urethra resulting in an increased bladder pressure required to empty the bladder. The result is an increased thickness of the bladder resulting from increased smooth muscle content and relatively normal functioning. This is called “compensated function” and most men are unaware of the changes in the bladder at this level. As men age and the prostate continues to enlarge, the pressure on the urethra reaches a level that the increased bladder mass cannot overcome and “decompensated function” is induced. Symptoms include a poor urine stream, incomplete emptying (increased residual volume), and in the final stages complete retention can occur [2, 3].

Relevance of the Rabbit Model of Outlet Obstruction to the Study of Human Bladder Dysfunction Secondary to Benign Prostatic Hyperplasia (BPH)

In humans, it is difficult to investigate the cellular mechanisms by which progressive obstructive bladder dysfunction occurs secondary to BPH. However, many of the functional changes associated with human bladder pathology can be induced in experimental animal models including the rabbit (see reviews [4-6]). Rabbit bladder capacity is between 50 and 100 ml and compliance can be evaluated cystometrically using an 8 Fr. Foley catheter to catheterize the bladder. The cystometric curve of the rabbit is similar in shape to that of humans: the bladder fills at low intravesical pressure until capacity is reached at which time the pressure rises sharply. Also similar to humans, bladder emptying occurs during the tonic

phase of contraction. The bladder's ability to sustain increased pressure in response to stimulation is significantly reduced by partial outlet obstruction before any change in maximal pressure generation occurs. This decreased ability to sustain increased pressure during stimulation is the reason that, in rabbits and humans, the bladder's ability to empty is reduced at times when the organ is capable of maximal pressure generation.

Major characteristics of the rabbit's response to partial outlet obstruction, i.e., an increase in bladder mass to a stable level, reduced compliance during bladder filling and development of overactive bladder syndrome (unstable bladder contractions during filling) in ~30% of obstructed animals [5, 7] are similar to those secondary to BPH in men. Ultrasound studies have confirmed that not only do men with obstructive uropathies exhibit an increase in bladder mass [8-11], but bladder wall thickness has been shown to be the most accurate noninvasive way to identify men with obstructive bladder dysfunction [8-11]. Another common feature of obstruction in both rabbits and man is denervation [4, 12, 13] which has been demonstrated immunohistochemically and biochemically in both species [4, 12]. In addition, obstructed rabbits and men both show an increase in the density and distribution of connective tissue (CT) within the bladder wall resulting, functionally, in decreased compliance and higher pressures during filling [14, 15]. We believe that these CT alterations contribute significantly to the contractile failure of the obstructed bladder [16-18].

In two major studies performed with Dr. John Gosling (expert in electron microscopy of the lower urinary tract), we clearly demonstrated that the level of contractile dysfunction in rabbits subjected to partial outlet obstruction correlated with the degree of ultrastructural damage to nerve, synaptic, mitochondrial, and sarcoplasmic reticulum (SR) membranes [12, 19, 20] which in turn correlates with similar findings in men with obstructive bladder dysfunction.

The Progression from Bladder Obstruction to Decompensation [21, 22]

The progressive response of the rabbit (and other animal species) to bladder dysfunction can be divided into three phases: 1) The initial response to partial bladder outlet obstruction (PBOO) lasts for approximately 14 days and consists of bladder distension and an increase in mass to a stable level. This increase in mass is associated with smooth muscle hypertrophy and mucosal hyperplasia. This phase does not correspond to human response to obstructive bladder dysfunction. 2) The second phase lasts a variable amount of time and consists of the bladder compensating for the obstruction and maintaining relatively normal contractile responses. At some point, the third and 3) final phase known as bladder decompensation sets in when the bladder loses its functional ability to contract and empty. This is a progressive deterioration of bladder function that will eventually lead to end stage decompensation. A bladder that has reached this end stage decompensation has little or no contractile function. However, the bladder may consist of either a thick fibrous wall with low capacity or be dilated with a thin fibrous wall and high capacity.

In the rabbit, the bladder maintains the ability to recover if the obstruction is removed even in the third phase as long as end stage decompensation has not been reached. The reversal will reverse the smooth muscle hypertrophy and mucosal hyperplasia, decrease the

bladder mass and restore the majority of the contractile function although perhaps not to the pre-obstructed state [23, 24].

Compensation versus Decompensation

Results of experiments in which mild partial outlet obstruction was studied longitudinally (up to 6 months) showed that the level of bladder decompensation was related to both the magnitude of the increase in bladder mass and the level of contractile dysfunction but not directly to the duration of obstruction. Therefore, we differentiate the status of the obstructed rabbit bladder (state of compensation/decompensation) by bladder mass and by the comparative contractile responses of isolated bladder strips to various forms of contractile stimulation [21, 25, 26].

Although it is clearly true that the longer rabbits are obstructed the greater proportion of them shift to decompensation and then progress to severe decompensation, individual rabbits may remain compensated or at mild decompensation for prolonged periods of time.

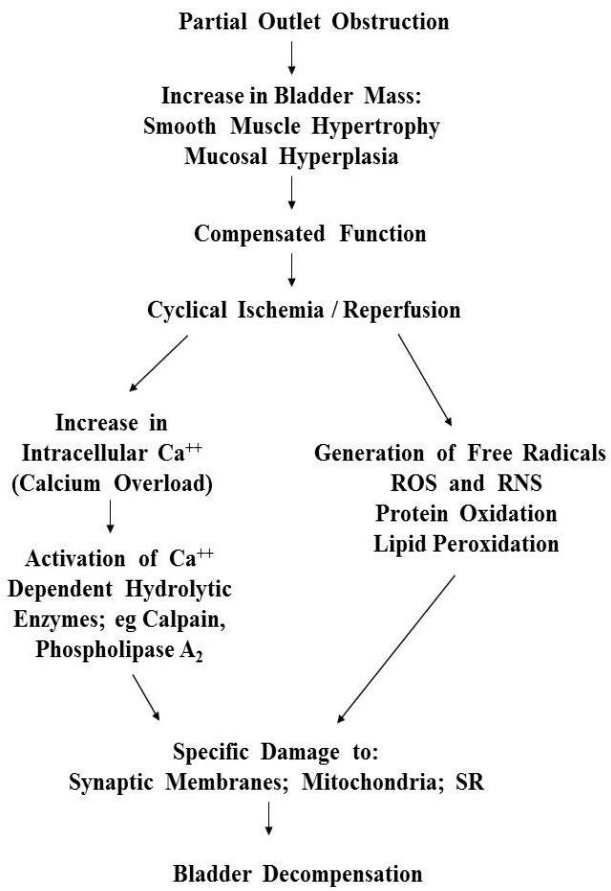


Figure 1. Schematic from Compensation to Decompensation.

This is very similar to progressive decompensation in men with obstructive bladder dysfunction secondary to BPH in that the level of obstructive dysfunction is not directly related to size of the prostate or how long individual men have had BPH. Some men with extremely large prostates have no urological problems while some men with small prostates have severe dysfunctions [2, 3].

The etiology of bladder dysfunction caused by PBOO in rabbits has been directly compared to obstructive dysfunction in men [4, 6]. In both obstructed men and rabbits, synaptic function is one of the most sensitive subcellular membrane systems to be damaged as indicated by significant decreases in the enzyme choline acetyltransferase (the enzyme that synthesizes acetylcholine in cholinergic synapses). A second very sensitive membrane system is the mitochondria as indicated by significant decreases in the marker enzymes citrate synthase and cytochrome oxidase. The third subcellular membrane system very sensitive to obstructive damage to men and rabbits is the sarcoplasmic reticulum (SR) which is responsible for uptake, storage and release of calcium required for contraction. We utilize the marker enzyme sarcoendoplasmic reticular ATPase (SERCA) as an indicator of the ability of the SR to uptake and store calcium following a contraction. Other similarities are the structural damage to these subcellular organelles observed under an electron microscope [12].

The following flow chart represents the progression from the compensated bladder function through decompensated function: (adapted from schematic from reference [27])

The first step for both men and rabbits is the initial response to urethral compression resulting in increased pressure required to empty the bladder efficiently. This is accomplished by smooth muscle hypertrophy which results in an increase in bladder thickness and mass [8, 9, 25, 28, 29]. The initial result is "compensated bladder function" which is characterized by nonsymptomatic bladder function in the presence of a mildly obstructed bladder. As the bladder wall thickens, every contraction of the bladder will compress the blood vessels within the bladder wall and induce cyclical ischemia (during the contraction) and reperfusion (immediately following the contraction) [5, 30, 31]. The ischemia/reperfusion can have two separate effects that result in significant damage to the bladder muscle and mucosa. Ischemia results in a reduced ability of the SR to take up and store the Ca^{++} that was released during the contraction resulting in "calcium overload" which is a significant increase in the intracellular free Ca^{++} that can stimulate specific calcium activated proteases and lipases such as calpain and phospholipase A_2 [32-34]. The result will be progressive damage to protein and lipid components of cellular and subcellular membrane systems such as neuronal, mitochondrial and SR membranes. These are the three most sensitive markers for obstructive bladder damage [4, 6, 14].

The second consequence of ischemia/reperfusion is mitochondrial dysfunction. Ischemia will significantly reduce the oxygen to the mitochondria resulting in a significant decrease in the ability of the mitochondria to generate ATP. The reperfusion (rapid increase in oxygen to the mitochondria) causes the generation of free radicals both reactive oxygen species (ROS) and reactive nitrogen species (RNS). This results is significant lipid peroxidation and protein oxidation [35-37]. The result will also be progressive damage to protein and lipid components of cellular and subcellular membrane systems such as neuronal, mitochondrial and SR membranes. As stated above, these are the three most sensitive markers for obstructive bladder damage [4, 6, 14]. The end result is progressive decompensation resulting eventually in full decompensation (retention).

It was of particular interest to better understand how the two sides of the pathway work in conjunction with one another; for example, discovering that one side predominated would dramatically improve the overall understanding of the biochemical changes associated with bladder decompensation. Fortunately, recent data has provided a tantalizing clue to the answer of this question.

In a series of studies, we obstructed rabbits for 4, 8, or 12 weeks and then evaluated bladder function according for both duration of obstruction or severity of dysfunction (mild, intermediate, or severe obstruction). In virtually all studies, we found that bladder physiological and biochemical dysfunctions were related to a significantly greater degree to severity than duration of obstruction. In all cases, the values of the obstructed rabbits were compared to sham operated rabbits and each other [32, 38, 39].

The severity was based on bladder weight: less than 6 grams was considered mild, 6-20 grams was seen as intermediate and over 20 grams was considered severe decompensation.

For the control rabbits (N=8) the mean bladder weight was 2.6 +/- 2.0 grams. For the 4 week obstructed rabbits (N=8) the mean bladder weight was 10.5 grams * and there were 3 mild, 3 intermediate and 2 severely decompensated rabbits. For the 8 week obstructed rabbits (N=8) the mean bladder weight was 16.4 +/- 4.8 grams * and there were 2 mild, 4 intermediate and 2 severely decompensated rabbits. For the 12 week obstructed rabbits (N=8) the mean bladder weight was 27.2 +/- 6.5 grams ** and there were 0 mild, 4 intermediate and 4 severely decompensated rabbits. * = significantly different from control; ** = significantly different from all other groups; $p < 0.05$.

Figure 2 shows the comparison of the contractile responses for the mild, intermediate, and severe decompensation with the free radical (oxidative stress) biomarkers nitrotyrosine (NT) (the product of free nitrogen radicals-RNS) and dinitrophenol (DNP) (the product of free oxygen radicals-ROS) [39]. In order to make comparisons easy visually, we normalized the contractile data to control = 100 since the response to obstruction was a decrease in contraction, whereas we normalized the free radical biomarkers to control = 10 since the response to obstruction was an increase in concentrations. There was a progressive decrease in the contractile responses to all forms of stimulation. Although we only show field stimulation and carbachol here, the responses to KCl and ATP were also quantitated. Please note that there was a significant decrease in all responses in the mildly decompensated group. However, there were not significant increases in either NT or DNP in the mildly decompensated group. For the intermediate and severe groups there were progressive decreases in the contractile responses which correlated with significant increases in both oxidative stress biomarkers [39].

Figure 3 shows the comparison of the activities of the calcium activated enzymes calpain and phospholipase A_2 (PLA_2) [32]. The data is normalized to control = 100% in order to make comparisons easy. For both calpain and PLA_2 there were significant increases in the mildly decompensated group. For calpain there was a continued increase in activity as decompensation progressed, whereas the PLA_2 activity remained elevated for the intermediate but decreased to near control levels for the severely decompensated bladders.

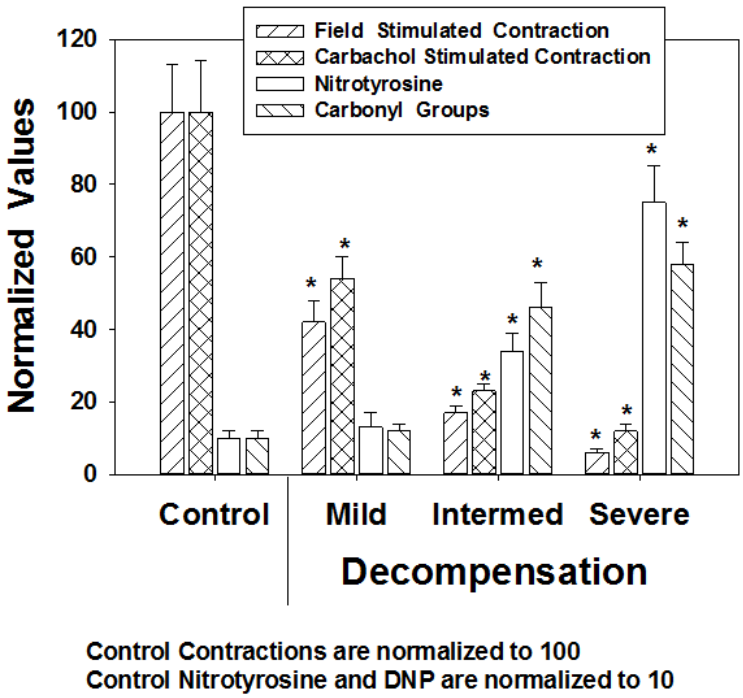


Figure 2. Correlation of the severity of bladder dysfunction on contractile responses to field stimulation and carbachol with the oxidative biomarkers nitrotyrosine and carbonyl groups. Each bar is the mean +/- SEM for between 4 and 8 individual rabbits. * = significantly different from control; $p < 0.05$.

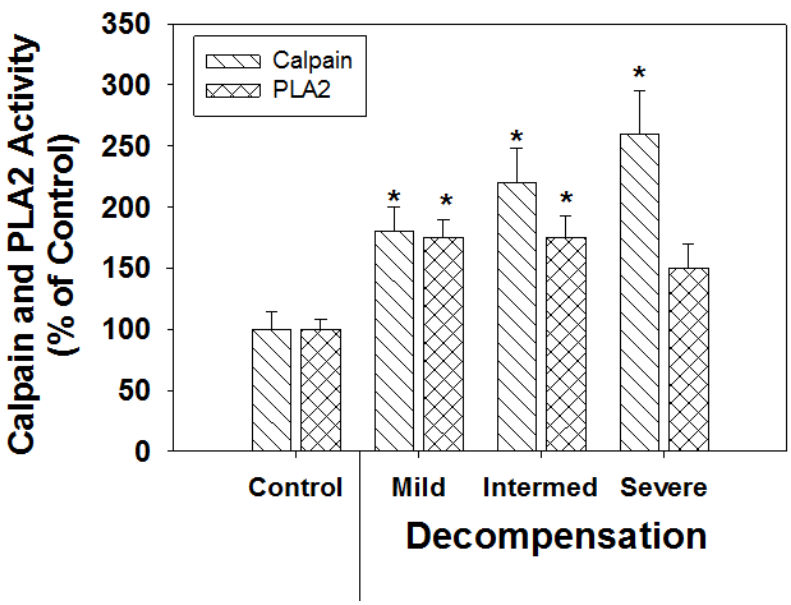


Figure 3. Correlation of the severity of bladder obstruction on the activities of calpain and phospholipase A_2. Each bar is the mean +/- SEM for between 4 and 8 individual rabbits. * = significantly different from control; $p < 0.05$.

The major conclusion from these studies was that mildly decompensated bladders may be better equipped to cope with oxidative stress than more severely decompensated bladders and that calcium overload may be the etiology of the contractile dysfunctions observed in the mildly decompensated bladders. Alternatively, it could be that the calcium dysregulation occurs sooner than the oxidative stress. Of course, a combination of the two possibilities is just as likely as either of the possibilities by themselves.

Connective Tissue As a Structural Impairment to Contraction

One of the major responses to PBOO is the conversion of smooth muscle to connective tissue notably collagen. In order to establish the relationship between the content and distribution of connective tissue to the contractile response of the bladder body we performed the following experiment [16]. Control and two week obstructed bladders were utilized for these experiments. The bladders were excised and strips were taken for both contractility studies and histological analysis. The relationship between strip length and contractile response was analyzed to determine if the density and distribution of collagen in the obstructed tissue was related to the contractile responses [16].

Figure 4 (Adapted from the figures in [16]) shows the relationship. In the control tissue, strip length did not affect the force per unit tissue mass. In the obstructed tissue, the shorter strips generated significantly more tension than the longer strips. This suggests that the collagen deposits were directly responsible for inhibiting optimal contraction. The histological analysis also showed the collagen deposits dispersed between the smooth muscle bundles in the obstructed bladders but not in the control bladders.

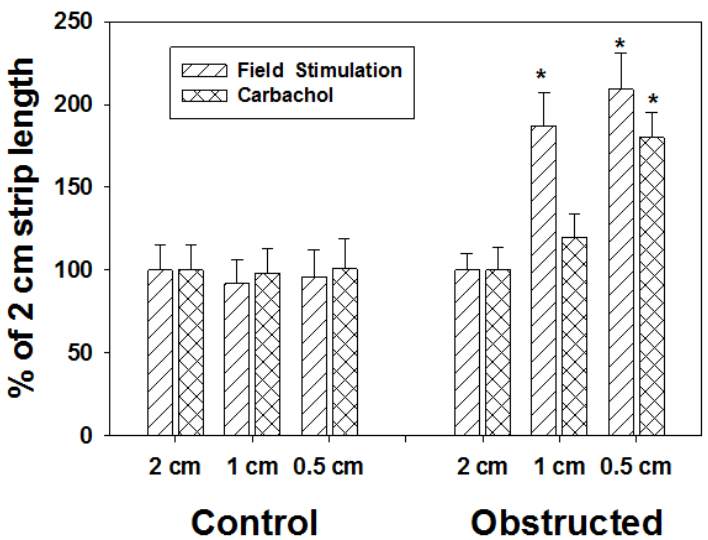


Figure 4. The effect of isolated strip length on the contractile responses to field stimulation (32 Hz) and carbachol for control and obstructed rabbits bladder bodies. Each bar is the mean +/- SEM for 4 individual rabbits. * = significantly different from 2 cm lengths; $p < 0.05$.

Bladder Obstruction Reversals

In several studies, bladder obstruction reversals were conducted to analyze to what degree bladders recover from decompensation and the various underlying mechanisms involved. This simulates men who undergo prostate reduction surgery. Interestingly, in virtually all of the studies bladder mass and function recover relatively quickly toward normal. The changes in bladder smooth muscle isoforms also return toward normal and the level of connective tissue also resolves [19, 24, 40-44]. Collagen may however follow a different course. In experiments where the obstructions were for a relatively short period of time (up to 8 weeks), the increased collagen is significantly reduced after reversal[43]. After prolonged obstruction when function can return toward normal, the changes in collagen are significantly less responsive [44]. It is known that with time, collagen can undergo structural changes in which cross linking occurs. This cross linking significantly reduces the effectiveness of collagenase (the enzyme that breaks down collagen), making reversal of the increase in collagen following reversal of obstruction more a function of duration of obstruction rather than the severity of dysfunction [45]. This may be an important factor in determining when men with obstructive bladder dysfunction should undergo prostate reduction surgery rather than be placed on medication.

Limiting Bladder Decompensation

As the various factors leading from bladder dysfunction to decompensation have been extensively discussed, the more practical questions are the ways to limit the damage caused by bladder dysfunction. The methods found to be successful in the rabbits would be important in that they would be well worth investigating in humans. Elucidating the process leading to bladder decompensation might lead the perceptive investigator to ask which methods are the best to slow down the cycle once it has been initiated by PBOO and the ensuing ischemia/reperfusion. The two pathways, oxidative stress and calcium dysregulation, are both important mediators of bladder decompensation. Studies have analyzed whether one predominates over the other. One recent important finding is that, as discussed above, in mild decompensation the calcium pathway seems to predominate over the oxidative stress.

Perhaps another way to evaluate the question is in the treatment of each pathway separately and then assessing if one is more beneficial than the other. For example, calcium channel blockers could be used to control the calcium dysregulation while antioxidants could be used to limit the oxidative stress. In this regard, there is substantial evidence that rabbits pretreated with both natural products with strong antioxidant properties and specific antioxidants can significantly reduce the progression of obstructive bladder dysfunction [46-50].

In summary, obstructive dysfunction in the male rabbit urinary bladder leading to decompensation has been well characterized and has proven to be an excellent model to investigate the etiology and prospective treatments for obstructive bladder dysfunction.

Acknowledgment

This material is based upon work supported in part by the Office of Research and Development of the Department of Veterans Affairs, and by the Capital Region Medical Research Foundation

References

[1] Steers, W.D., *Physiology of the urinary bladder, in Campbell's Urology*, A.B.R. P. C. Walsh, T. A. Stamey and E. D. Vaughan, Jr., Editor. 1992, Saunders Co.: Philadelphia. p. 142-176.

[2] Barry, M.J., Evaluation of symptoms and quality of life in men with benign prostatic hyperplasia. *Urology*, 2001. *58*(6 Suppl 1): p. 25-32; discussion 32.

[3] Barry, M.J.a.M., J.B., *The natural history of benigh prostatic hyperplasia, in Prostatic Diseases*, H. Lepor, Editor. 2000, Saunders Co.: Philadelphia. p. 106-115.

[4] Levin, R.M., et al., Obstructive response of human bladder to BPH vs. rabbit bladder response to partial outlet obstruction: a direct comparison. *Neurourol Urodyn*, 2000. *19*(5): p. 609-29.

[5] Levin, R.M., et al., Experimental Models of Bladder Obstruction., in *Prostatic Disease*, H. Lepor, Editor. 1999, W.B. Saunders Co: Philadelphia. p. 169 196.

[6] Levin, R.M., et al., Biochemical evaluation of obstructive bladder dysfunction in men secondary to BPH: a preliminary report. *Urology*, 1999. *53*(2): p. 446-50.

[7] Buttyan, R., M.W. Chen, and R.M. Levin, Animal models of bladder outlet obstruction and molecular insights into the basis for the development of bladder dysfunction. *Eur Urol*, 1997. *32 Suppl 1*: p. 32-9.

[8] Manieri, C., et al., The diagnosis of bladder outlet obstruction in men by ultrasound measurement of bladder wall thickness. *J Urol*, 1998. *159*(3): p. 761-5.

[9] Chalana, V., et al., Automatic Measurement of Ultrasound-Estimated Bladder Weight (UEBW) from Three-Dimensional Ultrasound. *Rev Urol*, 2005. *7 Suppl 6*: p. S22-8.

[10] Galosi, A.B., et al., Modifications of the bladder wall (organ damage) in patients with bladder outlet obstruction: ultrasound parameters. *Arch Ital Urol Androl*, 2012. *84*(4): p. 263-7.

[11] Rule, A.D., et al., Re: Ultrasound estimated bladder weight and measurement of bladder wall thickness-useful noninvasive methods for assessing the lower urinary tract? E. Bright, M. Oelke, A. Tubaro and P. Abrams. *J Urol* 2010;184:1847-1854. J Urol, 2011. *185*(6): p. 2435; author reply 2435.

[12] Gosling, J.A., et al., Correlation between the structure and function of the rabbit urinary bladder following partial outlet obstruction. *J Urol*, 2000. *163*(4): p. 1349-56.

[13] Nordling, J., Functional assessment of the bladder. *Ciba Found Symp*, 1990. *151*: p. 139-47; discussion 147-55.

[14] Mannikarottu, A.S., B. Kogan, and R.M. Levin, Ischemic etiology of obstructive bladder dysfunction: A review. *Recent Res. Devel. Mol. Cell Biochem.*, 2005. *2*: p. 15-34.

[15] Levin, R.M., et al., Etiology of bladder dysfunction secondary to partial outlet obstruction. Calcium disregulation in bladder power generation and the ability to perform work. *Scand J Urol Nephrol Suppl,* 1997. *184*: p. 43-50.
[16] Levin, R.M., et al., Effect of strip length on the contractile dysfunction of bladder smooth muscle after partial outlet obstruction. Urology, 2005. *66*(3): p. 659-64.
[17] Metcalfe, P.D., et al., Bladder outlet obstruction: progression from inflammation to fibrosis. *BJU Int,* 2010. *106*(11): p. 1686-94.
[18] Chaqour, B., et al., Cyr61 and CTGF are molecular markers of bladder wall remodeling after outlet obstruction. *Am J Physiol Endocrinol Metab,* 2002. *283*(4): p. E765-74.
[19] Malmqvist, U., A. Arner, and B. Uvelius, Contractile and cytoskeletal proteins in smooth muscle during hypertrophy and its reversal. *Am J Physiol,* 1991. *260*(5 Pt 1): p. C1085-93.
[20] Malmqvist, U., A. Arner, and B. Uvelius, Cytoskeletal and contractile proteins in detrusor smooth muscle from bladders with outlet obstruction--a comparative study in rat and man. *Scand J Urol Nephrol,* 1991. *25*(4): p. 261-7.
[21] Levin, R.M., et al., Genetic and cellular characteristics of bladder outlet obstruction. *Urol Clin North Am,* 1995. *22*(2): p. 263-83.
[22] Levin, R.M., et al., Effect of bladder outlet obstruction on the morphology, physiology, and pharmacology of the bladder. *Prostate Suppl,* 1990. *3*: p. 9-26.
[23] Wang, Z.E., et al., Expression of smooth muscle myosin isoforms in urinary bladder smooth muscle during hypertrophy and regression. *Lab Invest,* 1995. *73*(2): p. 244-51.
[24] Lin, W.Y., et al., Free radical damage as a biomarker of bladder dysfunction after partial outlet obstruction and reversal. *BJU Int,* 2008. *101*(5): p. 621-6.
[25] Kato, K., et al., The functional effects of long-term outlet obstruction on the rabbit urinary bladder. *J Urol,* 1990. *143*(3): p. 600-6.
[26] Nigro, D.A., et al., Metabolic basis for contractile dysfunction following chronic partial bladder outlet obstruction in rabbits. *Mol Cell Biochem,* 1999. *200*(1-2): p. 1-6.
[27] Levin, R., et al., Obstructive bladder dysfunction: Morphological, Biochemical and Molecular Changes. *European Urology Supplements*, 2002. *1*: p. 14-20.
[28] Mauroy, B., Bladder consequences of prostatic obstruction. *Eur Urol,* 1997. *32 Suppl 1*: p. 3-8.
[29] Bright, E., et al., Ultrasound estimated bladder weight and measurement of bladder wall thickness--useful noninvasive methods for assessing the lower urinary tract? *J Urol,* 2010. *184*(5): p. 1847-54.
[30] Greenland, J.E. and A.F. Brading, The effect of bladder outflow obstruction on detrusor blood flow changes during the voiding cycle in conscious pigs. *J Urol,* 2001. *165*(1): p. 245-8.
[31] Greenland, J.E., et al., The effect of bladder outlet obstruction on tissue oxygen tension and blood flow in the pig bladder. *BJU Int,* 2000. *85*(9): p. 1109-14.
[32] Callaghan, C., et al., The Effect of Partial Outlet Obstruction On Calpain and Phospholipase-2 Activity: Analyzed By Severity and Duration. *Molecular and Cellular Biochemistry,* 2013. *(In press).*
[33] Zhao, Y., et al., Correlation of ischemia/reperfusion or partial outlet obstruction-induced spectrin proteolysis by calpain with contractile dysfunction in rabbit bladder. *Urology,* 1997. *49*(2): p. 293-300.

[34] Hass, M.A. and R.M. Levin, The role of lipids and lipid metabolites in urinary bladder dysfunction induced by partial outlet obstruction. *Adv Exp Med Biol,* 2003. *539*(Pt A): p. 217-37.
[35] Kalorin, C.M., et al., Protein oxidation as a novel biomarker of bladder decompensation. *BJU Int,* 2008. *102*(4): p. 495-9.
[36] Juan, Y.S., et al., The effect of partial bladder outlet obstruction on carbonyl and nitrotyrosine distribution in rabbit bladder. *Urology,* 2007. *70*(6): p. 1249-53.
[37] Siflinger-Birnboim, A., R.M. Levin, and M.A. Hass, Partial outlet obstruction of the rabbit urinary bladder induces selective protein oxidation. *Neurourol Urodyn,* 2008. *27*(6): p. 532-9.
[38] Callaghan, C.M., et al., Effect of severity and duration of bladder outlet obstruction on catalase and superoxide dismutase activity. *Int J Urol,* 2013.
[39] Levin, R.M., et al., Partial outlet obstruction in rabbits: Duration versus severity. *Int J Urol,* 2013. *20*(1): p. 107-114.
[40] Nevel-McGarvey, C.A., et al., Mitochondrial and mitochondrial-related nuclear genetic function in rabbit urinary bladder following reversal of outlet obstruction. *Mol Cell Biochem,* 1999. *197*(1-2): p. 161-72.
[41] Stein, R., et al., The decompensated detrusor V: molecular correlates of bladder function after reversal of experimental outlet obstruction. *J Urol,* 2001. *166*(2): p. 651-7.
[42] Burkhard, F.C., et al., Contractile protein expression in bladder smooth muscle is a marker of phenotypic modulation after outlet obstruction in the rabbit model. *J Urol,* 2001. *165*(3): p. 963-7.
[43] Kim, J.C., et al., Effects of partial bladder outlet obstruction and its relief on types I and III collagen and detrusor contractility in the rat. *Neurourol Urodyn,* 2000. *19*(1): p. 29-42.
[44] Nielsen, K.K., et al., Morphological, stereological, and biochemical analysis of the mini-pig urinary bladder after chronic outflow obstruction and after recovery from obstruction. *Neurourol Urodyn,* 1995. *14*(3): p. 269-84.
[45] Furber, J.D., Extracellular glycation crosslinks: prospects for removal. *Rejuvenation Res,* 2006. *9*(2): p. 274-8.
[46] Agartan, C.A., et al., Protection of urinary bladder function by grape suspension. *Phytother Res,* 2004. *18*(12): p. 1013-8.
[47] Lin, A.D., et al., Protective effects of grape suspension on in vivo ischaemia/reperfusion of the rabbit bladder. *BJU Int,* 2005. *96*(9): p. 1397-402.
[48] Hydery, T., et al., Treatment of obstructive bladder dysfunction by coenzyme Q10 and alpha lipoic acid in rabbits. *LUTS,* 2013 (in press).
[49] Juan, Y.S., et al., The beneficial effect of coenzyme Q10 and lipoic acid on obstructive bladder dysfunction in the rabbit. *J Urol,* 2008. *180*(5): p. 2234-40.
[50] Parekh, M.H., et al., Protective effect of vitamin E on the response of the rabbit bladder to partial outlet obstruction. *J Urol,* 2001. *166*(1): p. 341-6.

Index

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y