A. Kumaresan
A. K. Srivastava *Editors*

Frontier Technologies in Bovine Reproduction

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Editors
A. Kumaresan
Theriogenology Laboratory
Southern Regional Station of
ICAR-National Dairy Research Institute
Bengaluru, Karnataka, India

A. K. Srivastava
Agricultural Scientists Recruitment Board
New Delhi, India

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Preface

Economic returns from bovine rearing, which depends upon individual animal lifetime productivity, are maximized when the reproductive efficiency is high. In the last century, dairy bovines were selected predominantly for milk yield at the expense of other traits including fertility. After realizing the importance of reproduction efficiency, off late, the fertility traits such as longevity and calving intervals have been included as a part of the selection traits. Although inclusion of these traits has served to reverse some of the earlier trends of declining fertility, because of low heritability of reproductive traits, improving lifetime reproductive efficiency through selection remains a challenge.

Calving to conception interval and reproductive longevity are two most important fertility parameters that determine success of bovine farming. However, analysis of existing information indicates that the reproductive longevity is reduced while the calving to conception intervals are increased, leading to reduced number of replacement heifers. It is documented that after three lactations, the incidence of reproductive disorders is high in high-yielding bovines. This situation requires a thorough understanding of the aetiological factors and application of promising technologies for restoring fertility. A book on "*Current concepts in bovine reproduction*" was brought out recently to update the readers with the recent developments accumulated in the area of reproduction management and to provide updated knowledge on concepts for improving reproduction efficiency in male and female bovines. In continuation, the present book on "*Frontiers Technologies in Bovine Reproduction*" is brought out focusing on the reproductive technologies that can play a vital role in restoring fertility in infertile/subfertile bovines and in improving herd reproductive efficiency.

In the recent past, reproductive biotechnology has taken a gigantic leap, and powerful reproductive technologies, such as oestrus induction, oestrus and ovulation synchronization, superovulation, embryo collection and transfer, *in vitro* embryo production, gamete cryopreservation, a variety of embryo micromanipulation procedures, and nuclear transfer, have been developed. This book provides updated information on reproductive technologies for fertility management and reproductive efficiency improvement both at individual animal and at herd level. Additionally, the recent technologies yet to be accepted widely but has potential in future for faster propagation of elite germ plasm are also discussed. Because male fertility is very

important in bovines bred artificially, technologies for identification of bull fertility are also covered in this book. We strongly believe that the book will be immensely useful to the students, researchers, and bovine farm managers in updating their knowledge on reproductive technologies for improvement of reproductive efficiency in bovines.

Bengaluru, Karnataka, India — A. Kumaresan
New Delhi, India — A. K. Srivastava

Contents

About the Editors

A. Kumaresan is as a principal scientist at Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru. His specialization is in veterinary gynaecology and obstetrics. He is a postdoc from Swedish Agricultural University, Sweden. His major area of research has been semen biology, sexing of semen, sperm–oviduct interaction and male fertility. He, along with his team, developed integrated livestock production models for hilly areas, area-specific mineral mixture for dairy cattle, and indigenous cryopreservation method for boar semen and produced the country's first litter of piglets through artificial insemination with frozen semen in 2009. His lab developed methods for in vitro culture of bovine spermatogenic and Sertoli cells, identified sperm fertility associated proteins and identified suitable combinations of sperm function tests for fertility prediction in cattle and buffalo bulls. In recognition to his significant contribution in research, Dr. A. Kumaresan has been awarded by ICAR with prestigious awards like Lal Bahadur Shastri Young Scientist Award (two times; one in 2007 and the other in 2015), Fakhruddin Ali Ahmed Award in 2010 and Hari Om Ashram Trust Award in 2019. In recognition of his teaching skills, NDRI awarded him with the "Best Teacher Award" for post-graduate teaching in 2014 and ICAR awarded him with "Bharat Ratna Dr. C. Subramaniam Award for Outstanding Teachers" in 2017. He is a recipient of BOYSCAST Fellowship by the DST in 2009, Dr. S. K. Sirohi Memorial outstanding researcher award in 2015 and Outstanding Research Faculty Award by Career 360 in 2018. He is a member of many scientific societies and organizations and fellow of the National Academy of Agricultural Sciences, National Academy of Dairy Sciences and Indian Society for Study of Animal Reproduction. He has published more than 225 research articles in peer-reviewed journals, 40 technical articles, 14 books and 9 bulletins besides several training manuals and book chapters. He has guided 17 master's students and 18 doctorate students as major advisor.

A. K. Srivastava is currently a member of Agricultural Scientist Recruitment Board. Previously, he was the director and vice chancellor at ICAR-National Dairy Research Institute, Karnal, dean, Faculty of Veterinary Science and Animal Husbandry, director, Resident Instructions, Dean PGs at Sher-e-Kashmir University of Agricultural Sciences and Technologies, Jammu, and head of the department at

Punjab Agricultural University, Ludhiana. He is postdoc from G.S.F Institute of Toxicology, Munich (Germany). Prof. Srivastava is president, National Academy of Dairy Science (India), and patron of Indian Dairy Association. He is president, Association of Mastitis, and president of Probiotic Association of India. Earlier, Prof. Srivastava was vice president of National Academy of Agricultural Sciences (NAAS), secretary, NAAS, and also the president, Indian Society of Veterinary Pharmacology and Toxicology. He is a Distinguished Member of National Academy of Sciences, fellow of National Academy of Agricultural Sciences, fellow of National Academy of Dairy Sciences and fellow of National Academy of Veterinary Sciences. He has been honoured with numerous prestigious awards and honours, including ICAR Jawaharlal Nehru Award, International NOCIL Award, National Alarsin Awards in 1987-88 and 1999-2000, ASET Gold Medal, German Academic Exchange Award, Gold Medals of Indian Science Congress Association, Dr. B.-D. Garg Outstanding Pharmacologist Award-2018, National Education Award 2016, and many more. He has been conferred the "Life Time Achievement Award" by the Indian Society for the Study of Reproduction and Fertility, Society for Community Mobilization for Sustainable Development and Indian Society of Buffalo Development. He has also received the "Chellappa Memorial Oration Award", Mansinhbhai Memorial Oration Award, Dr. Sunderasan Memorial Award, Dr. M. Sabir Oration Award, Prof. S.C. Mandal Memorial Oration Award, "Dr. Rao Memorial Oration Award", Dr. V. Kurien Memorial Oration Award and Prof. S. Kannaiyan Memorial Award-2019 by National Academy of Biological Sciences. He is honoured with Degree of Doctor of Science (D.Sc. Honoris Causa) from Veterinary University, Mathura, and Veterinary University Jabalpur. He has more than 250 research papers in his credit.

Introduction

1

A. Kumaresan and A. K. Srivastava

Abstract

Productivity largely depends upon reproduction, which in turn is influenced by several factors including genetic, nutritional, hormonal, physio-pathological conditions and management practices. The reproduction efficiency is determined by the combined effect of heredity and environment. Reproductive efficiency, generally, has a low heritability value indicating that most of the variations in this trait are due to non-genetic factors. Reproductive disorders and associated infertility (transient loss of fertility) among bovines pose serious economic loss to farmers in terms of low returns and veterinary expenses. Due to impaired reproduction ability, the calving to conception period is prolonged leading to extended calving interval, which jeopardize the aim of obtaining a calf per cow per year. In recent years, several reproductive technologies have been developed; the role of these "promising reproductive technologies" is immense in realizing the dream of "a calf per cow per year." This book details the information on reproductive technologies that could play an important role in restoring fertility in infertile/sub-fertile bovines, and provides updated knowledge on emerging technologies to improve reproductive efficiency in bovines.

Keywords

Bovine · Reproductive technologies · Fertility management · Fertility markers · Assisted reproduction

A. Kumaresan (✉)
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India
e-mail: A.Kumaresan@icar.gov.in

A. K. Srivastava
Agricultural Scientists Recruitment Board, New Delhi, India

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Bovine productivity has increased dramatically in the last few decades or so but at the same time, indices of reproductive efficiency have worsened. Across the globe, based on the analyses of large datasets, several studies have shown an inverse relationship of high productivity with reproduction efficiency. On the other hand, few researchers showed that high reproductive efficiency could be maintained in high producing bovine herds using proper management tools. Therefore, a greater understanding is required on the physiological basis of antagonistic relationship between milk production and fertility in bovines. Once the basis for the relationship is known, it is possible to identify the point of interventions in management strategies that can be implemented to counteract the negative effects of greater milk production on reproduction. It is expected that this approach would partially correct the decline in bovine reproduction efficiency. Therefore, it is evident that maximizing reproductive efficiency in bovines requires matching of genotypes to the production environment, together with appropriate husbandry practices, so that the intervals from calving to conception are short and the conception rates to natural or artificial breeding are high. In this regard, very recently, a book on "*Current concepts in bovine reproduction*" has been brought out with the aim to provide a suitable platform to the readers to gain an understanding of current concepts in the bovine reproduction starting from herd fertility trends and targets, molecules and mechanisms governing reproduction, novel approaches for oestrus detection, scope for improving reproduction efficiency using timed breeding protocols, emerging concepts on epigenetic bearing on fertility and semen cryopreservation and quality control. Interested readers may refer the book for a greater understanding on the concepts of bovine reproduction. This book on "*Frontier technologies in reproduction*" carries forward the concepts in the form of technology application for improving fertility in bovines.

Now, it is well understood that genetic selection for high milk production resulted in altered reproductive physiology of dairy cattle. Compared to traditional low producing dairy cows, modern dairy cows have longer intervals to first ovulation, a higher incidence of anoestrus and abnormal luteal phases, lower blood progesterone and IGF-I concentrations and greater embryonic loss. In high yielding animals, there is a rise in the energy demand to facilitate the dramatic increases in daily milk yield, which is partly met out by increased feed consumption, while the remainder is met by mobilization of body reserves resulting in animals entering negative energy balance (Grummer 2007). The negative energy balance predisposes the cows to several metabolic complications, immune compromised condition and reduced reproductive efficiency. It has been reported that up to 50% of modern dairy cows have abnormal postpartum oestrous cycles resulting in increased calving to first insemination intervals (Opsomer et al. 1998) and decreased conception rates (Dobson et al. 2008; Garnsworthy et al. 2009). Moreover, high producing dairy cows have shorter oestrus, less total standing time and lower serum oestradiol concentrations compared to low producing dairy cows (Lopez et al. 2004). Also, high producing cows are highly sensitive to heat stress contributing to the high rates of fertilization failure and embryonic mortality (Shehab-El-Deen et al. 2010; Fair 2010). A recent paper reports that over a period of time, the fertility decline was

evident in male bovines also (Kumaresan et al. 2021). In the given situation, reproductive technologies could play an immense role in restoring fertility in sub-fertile/infertile bovines and in improving herd reproductive efficiency.

Reproductive technologies were evolved through different phases of development and discoveries. In the 1980s, the results of artificial insemination (AI) from the previous decades were evident and, in the 1990s, the results of embryo transfer were evident. The commercialization of AI took place around 1950s after the methods for cryopreservation of semen were perfected. In several counties, more than 90% of the bovines are bred using AI technology. This technology necessitated identification and development of male fertility markers so that semen from high-fertile bulls is used in artificial breeding. Using high-throughput analytical techniques, new markers have been identified for male and female fertility. In the same manner as frozen semen, embryo freezing allowed for the global commercialization of animals with high genetic qualities. With the ability to produce in vitro embryos and to achieve pregnancies, the genetic manipulation of embryos and DNA became feasible. Currently, transgenic technology, which has origins through mechanisms or methods of over-expressing genes of interest or modulating gene expression, is gaining momentum. Collectively, in the last few decades, the field of bovine reproduction has expanded enormously, especially in dairy cattle and buffalos; advancements in the knowledge of reproductive physiology and improvements in analytical techniques have facilitated the development of several technologies for improving reproductive efficiency in bovines. A thorough understanding about the reproductive technologies is essential for their application in improving the reproductive efficiency of bovines. It is with this backdrop, this book is brought up with the aim to appraise the readers about the recent developments reproductive technologies and to elaborate their application in bovine breeding for restoring fertility in sub-fertile/infertile bovines and for improving herd reproductive efficiency.

Routine assessment of reproductive physiology and cyclicity is the foremost requirement for maintaining high reproductive efficiency in bovines. Accurate visualization of changes in the reproductive tract is now possible with the advent of non-invasive techniques like ultrasonography. The second chapter in this book details the use of ultrasonography in bovine reproduction management. This technology has opened new insights in better understanding of basic reproductive physiology as well as diagnosis of occult pathological conditions. This chapter explains the basics of ultrasonography, assessment of different reproductive organs and interpretation of results, and pregnancy diagnosis. This highly illustrative chapter also provides pictorial explanations and application of this technology in diagnosis of infertility in bovines. In continuation with ultrasonography, the third chapter provide an in-depth knowledge on application of infrared thermal imaging for reproduction management in bovines. This promising non-invasive and non-contact imaging technology can be used for remote monitoring of reproductive events. This chapter schematically explains the use of infrared thermal imaging for monitoring various reproductive events starting from oestrus to calving.

It also details the factors influencing thermographic imaging for logical interpretation of results.

The major step in reducing the calving interval is to identify the non-pregnant animals at the earliest and re-breed them. Routinely, in developing countries, rectal examination of cows at 45–60 days after breeding is employed for pregnancy diagnosis. In this method of pregnancy diagnosis, a significant proportion of empty cows after AI is declared as non-pregnant only after 45–60 days after AI leading to significant loss to the farmers. There is an ardent need for cow-side tests that can detect pregnancy/non-pregnancy by 16–20 days after AI. In the recent past, there has been a remarkable achievement in identification, characterization and validation of important pregnancy specific molecules using various biological samples for early diagnosis of pregnancy in bovines. The fourth chapter delineates the physiology of pregnancy and the potential molecules for pregnancy diagnosis besides the advancements in tools for early pregnancy diagnosis in bovines. Pregnancy associated changes in bovines, potential pregnancy specific molecules and advanced computer aided data collection and use in pregnancy diagnosis are also discussed in detail. It also discuss about merits and demerits of different techniques used for pregnancy diagnosis and also provide the way forward for developing early pregnancy diagnosis kits with high sensitivity and specificity.

The success of assisted reproductive techniques depends upon the quality of spermatozoa used. The sperm population within an ejaculate is heterogeneous, reflecting differing ability to fertilize an oocyte. Selecting sperm sub-populations with certain desired characteristics may have a positive effect on pregnancy rate in assisted reproduction. In this regard, chapters 5 and 6 detail the techniques that can be used to select spermatozoa with superior phenotypic and functional characteristics. Chap. 5 describes different sperm selection methods available with a special focus on the recently developed colloid centrifugation technology. Using this method, it is possible to remove bacteria from the spermatozoa, which is also detailed in this chapter. Chap. 6 describes a new technology for purification of semen using nanoparticles. The principle behind this nano-purification is to coat the iron nanoparticles with suitable antibodies or lectins for targeted capture and removal of desired or undesired cell populations. Several studies have shown that high quality spermatozoa can be enriched using this technique. Also, this chapter details about synthesis and characterization of nanoparticles, coating of nanoparticles with suitable molecules, and explains the positive or negative biomarker-based sperm purification.

Bull fertility is of paramount importance in bovines because semen from one bull is used for AI of several thousands of cows. Traditionally, breeding bulls are selected based on breeding soundness evaluation (BSE) that gives an idea about the mating and semen production ability of the bulls but not the fertility. Subsequently, a proportion of bulls that qualified BSE were found to be low-fertile bulls when they are used for artificial breeding. Therefore, a need is felt for identification of fertility biomarkers that can be used to select high-fertile bulls. Sperm function-based fertility prediction tools have been developed, but the results were not consistent. Off late, it was recognized that the molecular health of spermatozoa is

important for fertility and subsequent embryonic development. In the recent past, there has been a substantial development in technologies for assessment of sperm molecular profile, thereby leading to identification of sperm fertility markers. In chapters 7 and 8, high-throughput analytical tools for understanding sperm molecules in relation to fertility are discussed. Chap. 7 provides comprehensive understanding of sperm transcriptomic health in relation to fertility. The presence and roles of sperm mRNAs and importance of whole transcriptome profiling for sperm along with the detailed procedural advancements in the technique are discussed in this chapter. The utility of sperm mRNAs and microRNAs for male fertility prediction is also discussed in detail. Chap. 8 introduces the advances in sperm proteomics and metabolomics because these techniques have been widely accepted as the choice for identifying the fertility biomarkers in the sperm and seminal plasma. The details of the proteomic and metabolomic techniques and the typical workflow of these techniques for identification of fertility markers are clearly spelled out in this chapter. Additionally, information on the major proteomic and metabolomic biomarkers identified in sperm and seminal plasma are also discussed. Over the past two decades, spermatogonial stem cell transplantation has shown colossal potential for fertility restoration and transgenesis in livestock. Chap. 9 deals with the origin and self-renewal of spermatogonial stem cells and their transplantation for male fertility restoration. The advancements in isolation and enrichment of spermatogonial stem cells, their characterization, in vitro propagation and transfer techniques are also dealt in detail.

Embryo transfer is the most commonly used technology, just next to AI technology, for genetic improvement of bovines. Chap. 10 delineates the developments in the process and protocols for single and multiple ovulation embryo transfer. While explaining the benefits of embryo transfer in conservation and genetic improvement of animals, this chapter also deals with the drugs used in multiple ovulation embryo transfer and the species variations in superovulation response and success rates with embryo transfer. In continuation with multiple ovulation embryo transfer, the chap. 11 elaborates the process of ovum pick-up and in vitro embryo production in bovine. Different facets of this promising technology in reference to application, challenges, and future scope of the technology are also discussed in detail. In this chapter, a comparative understanding of in vivo versus in vitro embryo production systems, critical steps involved in in vitro embryo production technology and strategies to improve ovum pick-up efficiency in reference to in vitro embryo production are also explained in detail. Additionally, the current status of ovum pick-up and in vitro embryo production across the globe and the scope for this technology in genomic selection are also discussed.

Since the birth of the Dolly (world's first cloned farm animal produced from a differentiated adult somatic cell) in 1996, many technical and scientific developments have been made in animal cloning research worldwide, and over the years, several farm animal species such as cattle, goats, sheep, buffalo, pigs, horses, and camels have successfully been cloned. Recently, restoration of dead valuable bulls through cloning using donor somatic cells isolated from cryopreserved semen was carried out (Selokar et al. 2019) indicating animal cloning is an invaluable tool

to produce elite animals and for conservation of endangered breeds. Chap. 12 elaborates the animal cloning technology starting from the advantages of animal cloning, milestones in the technology, and recent developments in animal cloning as well as challenges and future prospective applications. A special note on buffalo cloning, production of genetically modified animals and therapeutic cloning is also provided in the chapter. Chap. 13 discusses about the advances and applications of transgenesis in farm animals. Recent developments in gene transfer techniques, including microinjection method, sperm mediated gene transfer, cytoplasmic DNA injection, somatic cell nuclear transfer, retroviral vector mediated methods, and embryonic stem cell-based methods are detailed in this chapter. Further, novel genome editing tools like designer nucleases for the generation of transgenic animals are also discussed.

In case of dairy bovine, the preference for female calves is high and in case of beef bovines, male offspring is preferred. During the recent past, several attempts have been made to develop a method that efficiently skew the sex ratio of offspring towards desired sex, including separation bovine semen into fractions containing higher concentrations of X- or Y-bearing sperm and modulation of female genital microenvironment. Chap. 14 narrates the different methods employed for skewing sex ratio of offspring towards either male or female along with their success rates. The concept of female control of offspring sex is also explained along with supporting theories.

With the advent of genome wide DNA markers, low-cost genotyping technology and development of suitable statistical methods, genomic selection came into picture. Chap. 15 explains the developments in genomic selection and how it can be applied for fertility improvement. The fertility traits that are to be considered for improvement, statistical methods for genomic prediction and genomic selection for male and female fertility traits are discussed in detail. Finally, the last chapter discusses about the prospects of stem cells in fertility management in bovines. The use of embryonic stem cells, induced pluripotent stem cells and adult stem cells in fertility restoration is detailed in this chapter. This chapter envisages that stem cell therapy could be a potential option in future for the management of reproductive disorders and enhancement of reproductive efficiency in cattle.

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Application of Ultrasonography in Bovine Reproduction

2

S. Satheshkumar

Abstract

With the introduction of improved concepts in breeding strategies (aiming at enhancing the production potential of the animals), an increase in infertility is obviously evident. It has posed new challenges among the farmers as well as the physicians. These challenges in bovine breeding systems warranted introduction of new user-friendly diagnostic technologies. Ultrasonography is a simple and potent technology that can strengthen the diagnostic range in the field conditions. With the help of ultrasound technology, it is possible to assess the entire reproductive system in a non-invasive manner. Reproductive ultrasonography is a dynamic introduction in the veterinary research and clinical investigation. Since 1980s, the ultrasonographic investigation has revolutionized the field of bovine reproduction. The technology has opened new insights in the better understanding of basic reproductive physiology as well as diagnosis of occult pathological conditions. The basic physical principles of ultrasonography, the proper use of the instrument's controls, the anatomical positions of the structures to be scanned, the interactions between the ultrasound wave and the organ tissue (the basis for obtaining quality images) and the interpretation of the obtained images appropriately applications of ultrasonographic investigation in reproductive physiology and pathology are discussed in detail in this chapter.

Keywords

Bovine ultrasonography · Reproductive cycle · Pregnancy · Pathology · Doppler ultrasonography

S. Satheshkumar (✉)
Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Thanjavur, Tamil Nadu, India

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Reproductive ultrasonography in bovines can be performed using sectorial or linear probe. Sectorial probe (trans-vaginal) doesn't require a large surface of contact, and it scans a greater overall surface and the latter (transrectal) provides good resolution for tissues located close to the probe. Transrectal linear-array transducer is easy to handle, animal friendly and can be used for more detailed study of utero-ovarian structures than the trans-vaginal probe. Hence, linear-array transducers are most commonly used in cattle for reproductive ultrasonography.

Depth of tissue penetration of sound waves and image resolution is dependent upon and inversely related to the frequency of the transducer. Thus, a 5.0 MHz transducer results in greater tissue penetration and lesser image detail, whereas a 7.5 MHz transducer results in lesser tissue penetration and greater image detail. The anatomical association of rectum and genitalia warrants transducers of 5–7.5 MHz frequency for the investigation of utero-ovarian structures.

The basics of ultrasonographic imaging depend on the echogenic or echoic nature of the particular organ or tissue (Fig. 2.1).

- An 'echogenic' or 'echoic' structure, such as bone and tissue, reflects the majority of sound waves back to the probe and thus appears from white to different shades of grey on the screen. Hypoechogenic and hyperechogenic refer to a decrease or an increase in relative echogenicity in comparison with the surrounding tissue, while isoechogenic refers to similar echogenicity with the surrounding tissue.

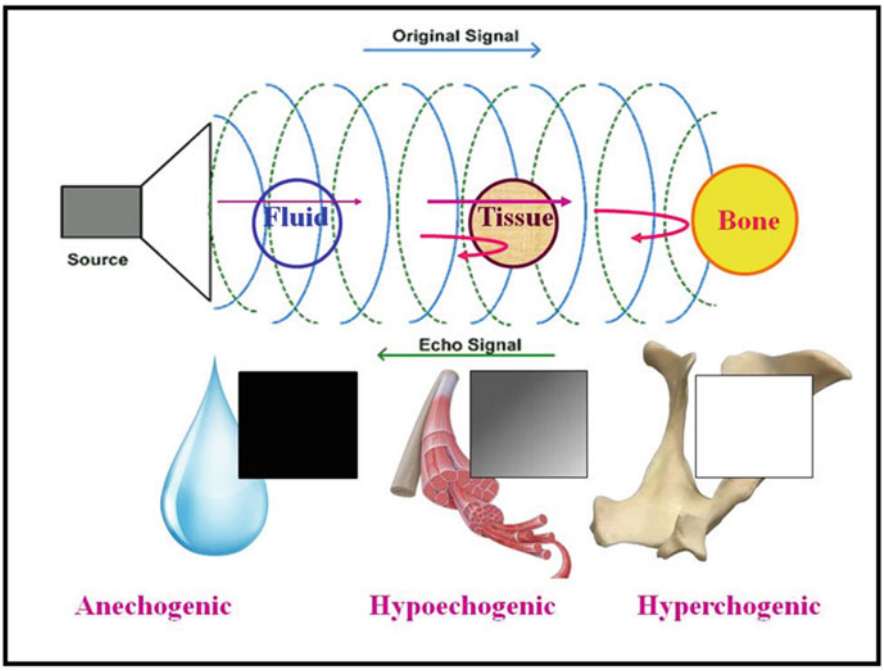


Fig. 2.1 Ultrasonographic imaging depends on the echogenic nature of the tissue

- An ‘anechogenic’ or ‘anechoic’ structure, such as fluid, does not reflect the sound waves, and the image appears black on the screen.

2.1 Ultrasonographic Imaging of Ovarian Structures

Transrectal ultrasonographic imaging is an effective tool for characterizing ovarian structures (ovarian parenchyma, follicle and corpus luteum) and tubular genital tract along with adnexa. It is non-invasive and permits repeated examinations of the utero-ovarian structures. Follicles that are below the ovarian surface and embedded CL, whose palpation by regular gynaeco-clinical examination is ambiguous, can be detected with precision using the ultrasonographic aids.

2.1.1 Imaging of Follicle

The most distinguishable ovarian structures are antral follicles. Antral follicles are fluid-filled structures and hence allow the ultrasound waves to pass through (i.e., anechoic or anechogenic) and are displayed as black images on the screen surrounded by hyper/hypoechogenic ovarian tissue (Fig. 2.2). Each ovary should be scanned and imaged in more than one plane to ensure that all measurable follicles were detected.

2.1.2 Imaging of Corpus Luteum

Corpus luteum (CL) appears as distinctly echogenic area within the ovarian stroma. The ovarian stroma and CL contain varying degrees of granular cells and result in a grey image on the screen. Usually CL appears as a solid tissue mass, but some CL may contain fluid-filled cavity (cystic corpora lutea) and the cavities range from <2 to >10 mm in diameter (Fig. 2.2). Embedded CL, which is often difficult to be palpated by per-rectal examination, can be documented clearly with the ultrasound technology.

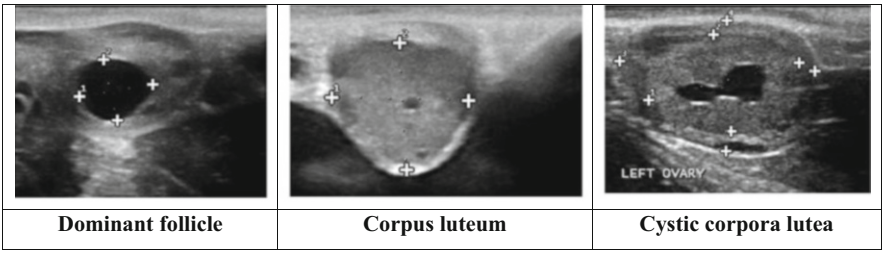


Fig. 2.2 Ultrasonographic imaging of follicle and CL. (**a**) Dominant follicle, (**b**) Corpus luteum, (**c**) Cystic corpora lutea

2.1.3 Imaging of Uterus

In a non-pregnant cycling cow, the uterine tissue normally appears as a partially echogenic structure on the screen. Since the uterus is comprised of soft tissue, it absorbs a portion of the ultrasound waves and reflects a portion of the waves. In this way, we can identify the uterus as a grey structure either in the cross section or sagittal section view on the screen (Fig. 2.3).

2.1.4 Biometry of the Utero-Ovarian Structures

The inbuilt electronic caliper of the ultrasound equipment can be utilized to measure the length and width of the ovarian structures, and the diameter was determined by taking the mean of these two values (Savio et al. 1988). Based on the ultrasonographic studies in crossbred cattle over years, certain biometrical standards were arrived for clinical assessment of the structures, as follows.

1. Follicles with diameter of >8.5 mm are considered as dominant follicle (DF).
2. CL with diameter of >15.0 mm is considered as mature and active.
3. CL with diameter of <15.0 mm is considered as regressed structure.

Likewise, the thickness of uterine endometrium, embryonic/foetal dimensions, etc. can be measured in a linear fashion using the electronic caliper in the ultrasound equipment.

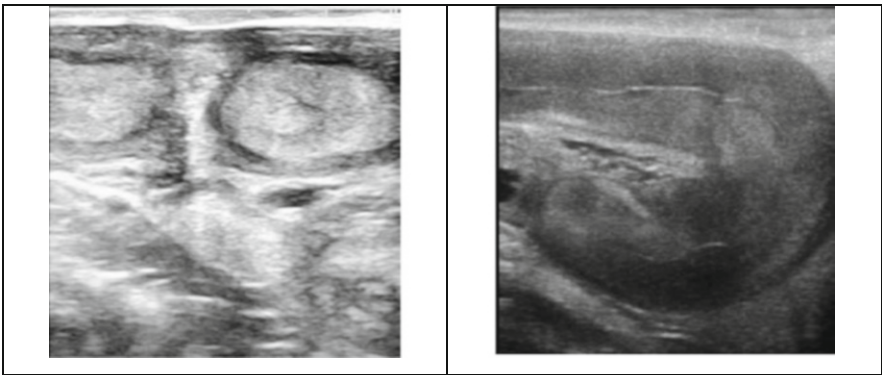


Fig. 2.3 Ultrasonographic imaging of non-pregnant uterine horns—cross section and sagittal section view

2.2 Ultrasonographic Characterization of Folliculogenesis and Luteal Development

In cattle, ovulatory follicles do not develop randomly, but their development occurs in a regular fashion. For better understanding of ovarian physiology, various investigative techniques have been used to characterize the follicular development in cattle including the histological evaluation of ovaries, the correlation of follicular inventories with measurement of steroids in follicular fluid and laparoscopic monitoring of individual follicles by marking them with dyes. The studies and advancements that have led to our current understanding regarding patterns of follicular development are listed below in chronological order:

1960 The two-wave concept for follicular growth during the bovine oestrous cycle was proposed (Rajakoski 1960).

1972 and 1981 The lifespan and fate of individual follicles during the oestrous cycle of heifers were directly examined (Dufour et al. 1972; Matton et al. 1981).

1982–1983 Growth and differentiation of oestrogen-active and oestrogen-inactive follicles during the oestrous cycle were distinguished (Ireland and Roche 1982, 1983).

Although these earlier studies provided important information, they precluded the sequential monitoring of individual follicles over a long duration, which led to conflicting ideas about follicular dynamics in cattle. With the advent of real-time ultrasonography, it became possible to visualize the various reproductive events in large animal species, especially cattle, over a prolonged period of time and without any interruption of normal physiological events. The modern era of study of follicular dynamics in cattle began with the classic publication by Pierson and Ginther in the year 1984.

1984 Ultrasound to monitor sizes of follicles during the oestrous cycle of heifers was used (Pierson and Ginther 1984).

1987 The concept of DFs, as observed in primates, was applied to cattle, and the three-wave hypothesis for development of DFs during the oestrous cycle was proposed (Ireland 1987).

1988 Ultrasound analysis and ovarian maps to track growth and atresia of individual follicles throughout the oestrous cycle of heifers were used (Savio et al. 1988; Sirois and Fortune 1988).

Since 1984, the application of real-time ultrasonography in monitoring the utero-ovarian function took off in a jet propulsion and has advanced the understanding of follicular and luteal dynamics during the cycle.

2.2.1 Ovarian Follicular Dynamics

Based on the preliminary ultrasonographic documentation, Pierson and Ginther (1984) recorded that there was (1) growth of a large follicle to an ostensibly ovulatory size followed by regression at approximately mid-cycle, (2) selective accelerated growth of the follicle destined to ovulate approximately 3 days before

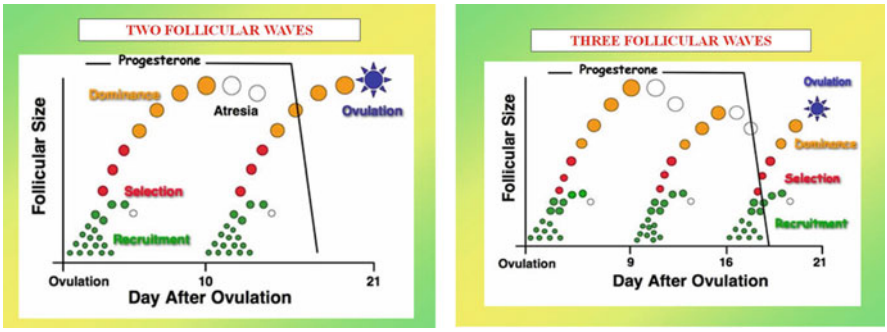


Fig. 2.4 Different follicular wave patterns during the oestrous cycle

ovulation and (3) a few days before ovulation regression of the larger follicles that were not destined to ovulate.

Further investigations have proved that folliculogenesis operates on a wave-like fashion at 7–8 day intervals throughout the oestrous cycle in *B. taurus* (Ginther et al. 1989a, b; Pursley et al. 1993), *B. indicus* (Viana et al. 2000; Gaur and Purohit 2007) and *B. taurus x B. indicus* crossbred (Satheshkumar et al. 2008a, 2011, 2012a; Satheshkumar 2015a) cattle.

Follicular wave is a sequence of organized events consisting of the synchronous growth of small (4–5 mm) antral follicles (recruitment), followed by the selection, deviation and growth of one dominant follicle (DF) which achieves the largest diameter and suppresses the growth of the subordinate follicles. In the absence of luteal regression, the DF eventually regresses (becomes atretic) and a new follicular wave begins in a day or two. The DF regulates the growth of the subordinate follicles because the appearance of the next wave is accelerated if the DF is ablated, and delayed if the lifespan of the DF is prolonged. These sequence of events comprising of recruitment, selection, deviation, growth, dominance and regression of a DF is known as a follicular wave. During bovine oestrous cycles, two or three successive waves emerge, on average, on the day of ovulation (day 0) and day 10 for two-wave cycles, and on days 0, 9 and 16 for three-wave cycles (Duggavathi et al. 2003). Thus, there two or three successive DFs develop during an oestrous cycle, and the last of these ovulates with the advantage of luteolysis (Fig. 2.4).

Two and three follicular wave patterns are common among the Indian crossbred cattle but their incidence during an oestrous cycle is highly variable and unpredictable (Satheshkumar et al. 2008a, b; Satheshkumar 2015a, b). These variations in follicular wave patterns are one of the major contributory factors of fertility disturbances among the dairy cattle population. It was reported that oestrous cycles with three follicular waves have better fertility than those with two follicular waves (Townson et al. 2002). Various factors govern the follicular wave pattern during an oestrous cycle like the plane of nutrition (Murphy et al. 1991), circulating endocrine status (Adams et al. 1992), season (Satheshkumar et al. 2015), luteal status (Satheshkumar 2020), etc.. However, the basic ultrasonographic studies have

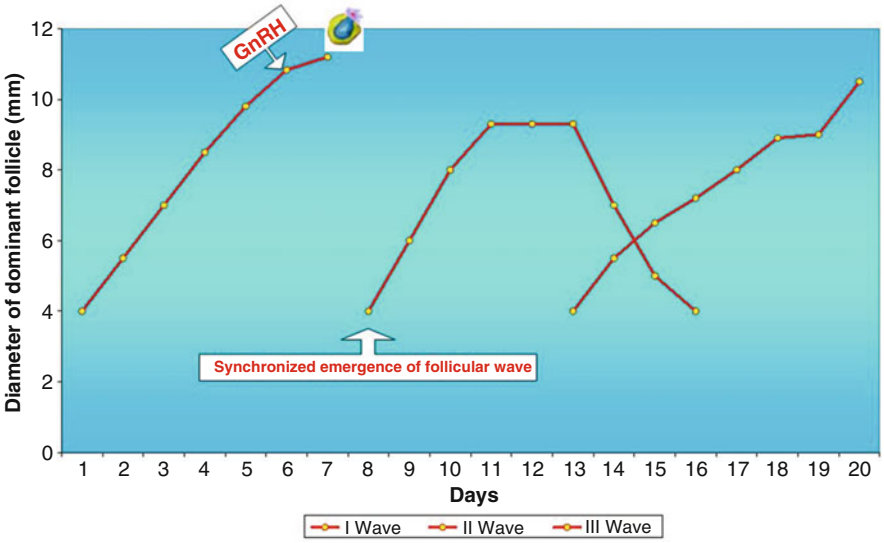


Fig. 2.5 GnRH induced occurrence of three follicular waves

given the concept for inducing the occurrence of three follicular wave cycles by hormonal intervention (Satheshkumar et al. 2012a, b). When GnRH analogue (Buserelin acetate–10 μg) was administered on Day 5 or 6 of the cycle, the DF of first follicular wave ovulated and a synchronized homogenous group of follicles emerged after 2 days. Because of early emergence of second follicular wave and high progesterone (P_4) concentration in the mid-cycle due to accessory CL formation, the DF of second follicular wave will regress and eventually the third wave arise (Fig. 2.5). Thus, positive manipulation of follicular wave emergence is made possible because of better understanding of follicular physiology.

2.2.2 Ovarian Luteal Dynamics

Fertility of any mammalian species is closely related to their luteal activity. In cattle, CL is the prime source for P_4 synthesis and hence its sustained function needs to be secured in order to support pre-implantation embryonic development and to maintain the pregnancy (Roberts 1971; Grana-Baumgartner et al. 2020). Ginther et al. (1989a, b) reported that the time of luteal regression is one of the factors that determine the number of follicular waves during a cycle. The pattern of luteal development was sequentially drafted using the transrectal ultrasonography in crossbred cattle (Satheshkumar 2020). In general, the CL grew progressively until it attained the maximum diameter (21.7 ± 0.4 mm) during the mid of the cycle (mean day 9.5 ± 0.4) and remained fluctuating around that size for a certain period (period of luteal dominance; 5.7 ± 0.6 days) before it started regressing constantly (from Day 15.2 ± 0.8) towards the end of the cycle.

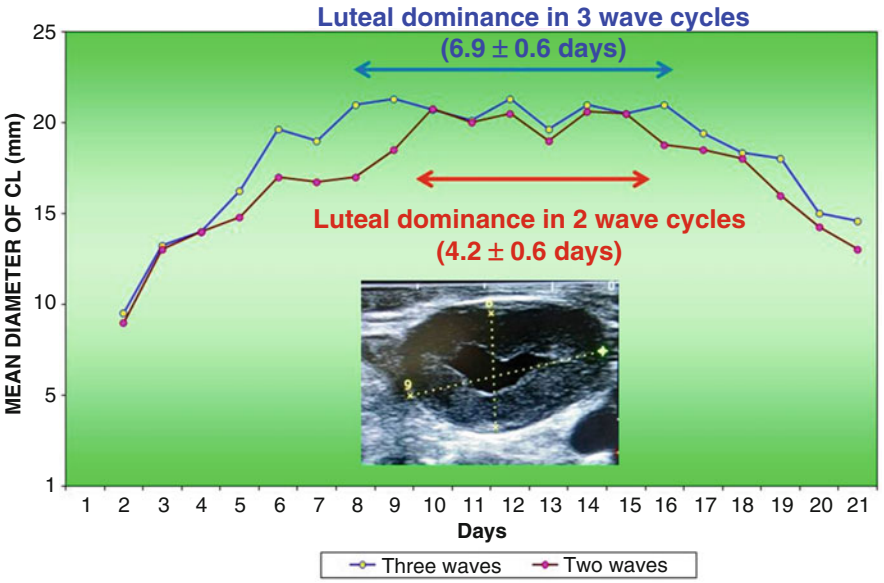


Fig. 2.6 Luteal dynamics during two- and three-follicular wave cycles

The variations in luteal development characteristics were observed in oestrous cycles with two follicular waves and three follicular waves (Fig. 2.6). The maximum diameter of CL was significantly larger in three-wave cycles than that of two-wave cycles. In cycles with three follicular waves, the CL reached the maximum diameter significantly earlier, remained dominant for a significantly longer duration and started to regress non-significantly later when compared with cycles of two follicular waves. The early attainment of maximum diameter and late regression of CL during a three-follicular wave cycle assured a significantly increased period of luteal dominance than the two-follicular wave cycles. Thus, it could be concluded that the period of luteal dominance and the associated luteal endocrine activity have a possible control over follicular turnover during spontaneous oestrous cycle in crossbred cows. Taking into consideration of the importance of luteal support on embryonic sustenance and establishment of pregnancy, it is very obvious to associate the luteal insufficiency and prolonged persistence of second wave DF with the poor fertility in cows with two-follicular wave cycles.

2.3 Role of Ultrasound in Diagnosing Physiologic Reproductive Events and Its Variations

The reproductive efficiency of an animal is influenced by many extrinsic factors such as nutrition, lactational status, season, breeding practices, etc. However, recent epidemiological studies indicate that intrinsic factors like variations in follicular

and luteal development contribute to the fertility disturbances in dairy herds. Ovarian follicles are the vital units which provide proper environment and nourishment for the female gametes, the oocytes and contribute for the emergence of healthy CL. The endocrine status of both these structures provides a protective uterine environment for the establishment of pregnancy. Any deviation in this utero-ovarian chain during the oestrous cycle can directly or indirectly affects the fertility of the animal. In addition to the routine gynaeco-clinical examination, the ultrasonographic investigation provides a confirmatory aid to assess the utero-ovarian characteristics in cattle and to manipulate it positively for enhancing the fertility.

2.3.1 Diagnosis of Oestrum and Its Aberrations

Oestrus is the most precious phase of oestrous cycle and is the gateway for fertility. Efficient oestrus detection is a key factor that determines the success of breeding program in a dairy herd. In spite of progress in the development of oestrus detection aids, errors occur leading to setback in reproductive management of dairy cows. Variations in the duration and intensity of oestrus warrant the need for accurate and continuous monitoring to determine the optimal breeding time. The low accuracy and efficiency of oestrus detection not only increases time from calving to first AI but also increases the average interval between services (Stevenson and Call 1983), thereby limiting the overall pregnancy rate.

In the field conditions, whether all the animals are being inseminated in the actual oestrus? Nebel et al. (1987) reported that insemination of cows not in oestrus was a problem in 30% of the herds and emphasized that errors in detection of oestrus must be considered as a significant cause of low conception rates in problem herds. The behavioural changes, clinical signs, and per-rectal gynaeco-clinical investigation of uterine status were considered to be the important approaches in diagnosing oestrus in cattle, but it is quiet astonishing to know the fact that many animals are being inseminated in the wrong time of the oestrous cycle with these routine diagnostics.

Anoestrus, prolonged oestrus and silent oestrus are the widely discussed aberrations of oestrus expression pattern in cattle, but in the recent years occurrence of 'mid-cycle oestrus' (MCO) is found to be emerging as a serious aberration in the crossbred cows (Sood et al. 2009; Satheshkumar et al. 2014a, b; Satheshkumar et al. 2019a, b). The exhibition of oestrus-like signs during the early or mid-luteal phase (Day 6–12) of the cycle is referred as MCO. Apart from external signs like restlessness, vaginal discharge, mounting, etc., routine gynaeco-clinical examination also revealed moderate to good tonicity of uterine horns simulating the oestrus. These factors mislead the farmers and the veterinarians equally in diagnosing the oestrus. Sood et al. (2009), Satheshkumar et al. (2014a) and Satheshkumar (2018a) recorded the incidence of MCO among crossbred cattle as 2.4, 6.2 and 8.8%, respectively. Perusal of these reports revealed that the incidence of MCO is in increasing trend and is also equally limiting the reproductive outcome in cattle.

Luteal deficiency is the major factor contributing to the occurrence of MCO (Satheshkumar 2018a). The increased oestradiol (E_2) concentration secreted by the

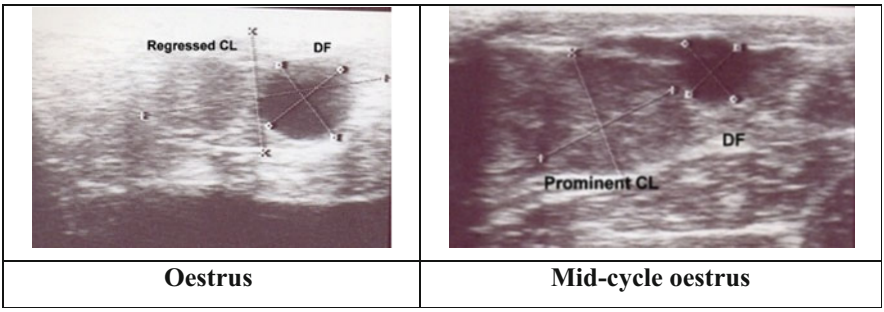


Fig. 2.7 DF and CL status during oestrus and MCO (a) Oestrus, (b) Mid-cycle oestrus

DFs of first or second follicular wave overrode the P_4 and induced the animal to exhibit oestrus-like signs during the mid-cycle. Animals won't conceive when inseminations are carried out during MCO because P_4 concentration during diestrum is sufficient enough to prevent the ovulation. Further, there is every likelihood of inducing embryonic mortality by inseminating the animal that would have conceived to previous insemination which was done 7–10 days back. Number of inseminations per conception tends to increase when animals are inseminated repeatedly during MCO, thereby falsely projecting the incidence of repeat breeding condition.

With the application of ultrasonographic imaging technology, animals in proper oestrus are diagnosed with more precision. Before proceeding for insemination based on routine clinical examination, ultrasonographic assessment of DF and CL characteristics provides the exact reproductive status of the animal. How? Biometric assessment revealed that the mean diameters of DF and regressed CL during oestrus were 11.9 ± 1.5 mm and 12.7 ± 1.7 mm, respectively. However, the corresponding values in MCO were 10.9 ± 1.1 mm and 19.3 ± 1.0 mm, respectively. There was no significant difference in the diameter of largest follicle present during oestrus and MCO, but the diameter of CL was significantly larger in MCO (matured CL during diestrum) than oestrus (Satheshkumar 2018a). Hence, based on ultrasonographic examination, a basic thumb rule can be followed before deciding to perform insemination in the animal (Fig. 2.7).

- A combination of a DF (with >8.5 mm diameter) and regressing CL (with <15.0 mm diameter) indicates oestrus. Animal can be inseminated.
- A combination of a DF (with >8.5 mm diameter) and prominent/mature CL (with >15.0 mm diameter) indicates the MCO. Insemination should be avoided.

Assessment of CL size by ultrasonographic examination should be practiced regularly to distinguish between oestrus and MCO, thus inseminations at correct stage of the cycle are ensured.

2.3.2 Diagnosis of Ovulation and Its Disturbances

Ovulation is an important physiological event that determines the fertility of an animal and any disturbance in this event will result in anoestrum, cystic degeneration or repeat breeding syndrome. Normally ovulation occurs 10–12 h after the end of oestrum in cattle. In the recent years, we could observe lot of disturbances in the ovulatory processes, especially delay in the timing of ovulation which is evidenced as prolonged oestrus.

A sequential ultrasonographic study (12 h interval) of the DFs from the onset of oestrus gave a clear indication about the incidence of ovulatory disturbances in crossbred cattle (Satheshkumar 2018b). Ovulation was confirmed when the DF was no longer seen in the same location on subsequent examination (Kim and Kim 2007). The follicular size increased non-significantly until the last examination prior to ovulation. It was found that ovulation occurred between 24 and 36 h after onset of oestrus in 54.6% of cycles (normal) and between 36 and 60 h after onset of oestrus (delayed) in the rest of the cycles (45.4%). Thus, delay in ovulation seems to be a major setback in the fertility of dairy cattle. Based on this ultrasonographic documentation, double inseminations at 24 h interval are being recommended in all the oestrus animals to ensure better conception rate.

2.3.3 Diagnosis of Pregnancy

Early pregnancy diagnosis is a key factor in the reproductive management of dairy herd. Timely diagnosis of the non-pregnant status of an animal is essential to rebreed at the subsequent oestrus and to maintain an optimum calving interval. A variety of methods were designed to identify the pregnant animal in the earlier stages.

1. *Indirect Methods* include hormonal, immunological, and chemical approaches.
2. *Direct Methods* include transrectal palpation and ultrasonography.

In human, hormonal estimation has been standardized for early diagnosis of pregnancy. In animals, especially in cattle, lots of pregnancy associated biomarkers are being identified but there lies an ambiguity in precise confirmation of pregnancy till date. Hence, direct methods are found to have advantage over indirect methods in the current scenario. Among the direct methods, the non-pregnant animals can be identified at least 15–30 days earlier by using ultrasonography when compared to standard transrectal method of examination.

A minimum of 60 days in the post-breeding period is required to diagnose pregnancy through rectal palpation. However, experienced and skilled veterinarians can confirm pregnancy at an early stage of 40–45 days post-breeding by the palpation of amniotic vesicle and double-slipping of chorioallantoic membrane. Several studies have suggested that examining pregnant cows early in gestation, especially by inexperienced technicians increases the risk of iatrogenic embryonic mortality. While rectal palpation is reliable and a widely accepted method for

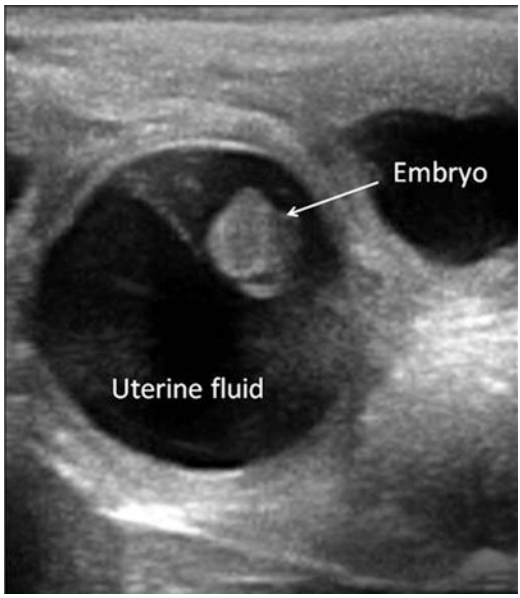


Fig. 2.8 Ultrasonographic imaging of uterine fluid and embryo by 24 days post-insemination

pregnancy diagnosis, it is difficult to assess embryonic viability and/or detect pregnancy loss with this technique.

Positive diagnosis of pregnancy is possible as early as 25 days post-insemination by the application of ultrasonography. Non-regressed CL (>15 mm diameter) by the period between 20 and 25 days post-insemination can be considered as the first indicator of pregnancy; however, it is not confirmatory due to various pathological conditions. As the conceptus develops, fluid accumulates within the allantoic and amniotic cavities during early gestation. Careful ultrasonographic examinations in the tip of the uterine horn, ipsilateral to the ovary having the CL, enable the examiner to identify this fluid accumulation as anechogenic zone along with the embryo (Fig. 2.8). Early pregnancy diagnosis using ultrasonography shows the uterine lumen containing a variable amount of. Identification of prominent CL and uterine fluid by 20–22 days and embryonic mass with heartbeat between 24 and 30 days post-breeding are considered as positive signs of pregnancy in cattle. These features make the ultrasonographic intervention a reliable method of early pregnancy diagnosis in cattle.

Scanty fluid accumulation could be observed in the uterine horn during oestrus also, due to physiological secretions and it should not be mis-diagnosed as pregnancy. How to differentiate it from early pregnancy? During oestrus, the mucus membrane folds are evident in the uterine endometrium of crossbred cattle, but the fluid-filled lumen of pregnant uterine horn possesses smooth lining without any 'crenulation like' pattern (Fig. 2.9).

If an animal is presented for insemination with oestrus signs, which was inseminated in the previous cycle, it should be subjected for ultrasonographic investigation. If anechogenic fluid with smooth luminal wall could be observed in

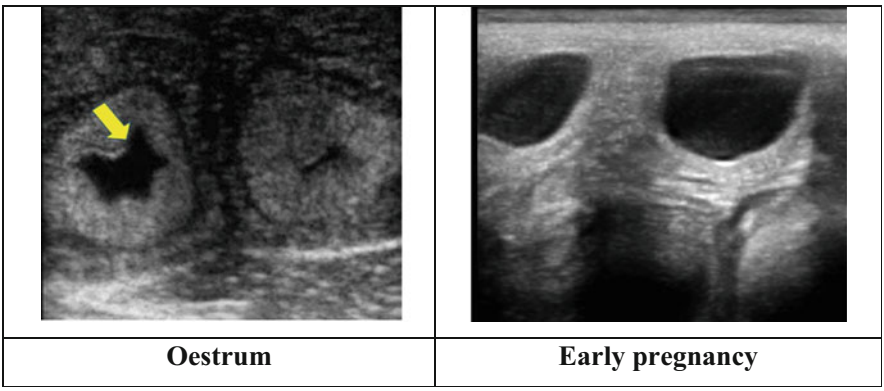


Fig. 2.9 Uterine endometrial pattern during oestrum and early pregnancy. (**a**) Oestrum, (**b**) Early pregnancy

the tip of any uterine horn along with a prominent CL in the ipsilateral ovary, it should be suspected for early pregnancy and insemination should be avoided. Sometimes early pregnant animals evince oestrus signs due to presence of DF at that time. If such animals are inseminated, we will probably lose the pregnancy due to accidental induction of embryonic mortality. This might be one major reason for pregnancy loss in field conditions, where ultrasound examination is not practiced.

- The importance of ultrasonographic investigation of utero-ovarian structures before attempting for insemination in animals with oestrus signs is reiterated.

Mucometra and pyometra are generally defined as an accumulation of mucus and pus within the uterus, respectively. Compared to manual palpation, the differences between uterine enlargement due to pregnancy and these pathological conditions are easily recognizable on ultrasound examination. While fluid in the pregnant uterus appears anechoic, pyometra appears as distension of the uterine lumen with contents of mixed echogenicity. In case of mucometra, uterine fluid appears clear and anechoic like that of pregnancy, but there are no evidence of foetus, foetal membranes or placentomes on ultrasound examination (Fig. 2.10). Thus, ultrasound technology is the reliable method of diagnosing pregnancy and also to differentiate it from other pathological conditions.

2.3.4 Assessment of Gestational Age

Two methods can be used to determine the gestational age of the embryo or foetus. First method is based on direct measurements of the embryo or foetal dimensions, and the second method is based on the first appearance of certain embryonic or foetal structures.

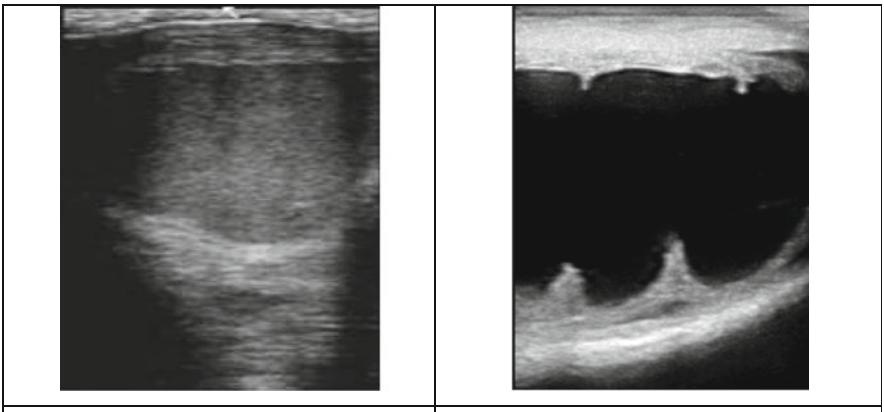


Fig. 2.10 Ultrasonographic imaging of mucometra and pyometra (**a**) Pyometra, (**b**) Mucometra

Ultrasonographically, four different measurements can be made based on the view of the embryo/foetus. They are crown–rump length (CRL), trunk diameter, head diameter and head length.

- CRL is measured from the tail head to the crown of the skull.
- Trunk diameter is the widest part of the ribcage at the level of the umbilicus.
- Head diameter is the maximum diameter of the skull just caudal to the eyes.
- Head length is measured from the top of the cranium to the tip of the nose.

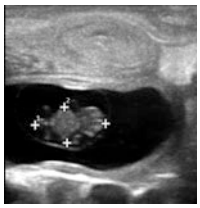
CRL is the earliest possible measurable parameter before the head and trunk portions can be distinguished. Until 50 days of gestation, a simple formula can be applied for assessing gestational age as follows:

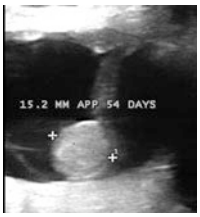
$$\text{Length of the Embryo or Foetus (Mm)} + 18 = \text{Embryonic or Foetal Age (Days)}$$

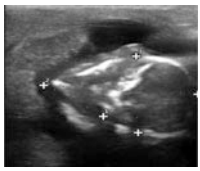
For foetal ages greater than 50 days, 'gestational chart' should be used (Table 2.1). Some ultrasound units are equipped with inbuilt software to calculate the gestational age based on foetal biometry.

Curran et al. (1989) standardized the assessment of gestational age based on visual evaluation of first appearance of certain embryonic/foetal features, as mentioned in Table 2.2.

Table 2.1 Gestational chart for cattle







Crown–rump length		Trunk diameter		Head diameter		Head length	
mm	days	mm	days	mm	days	mm	days
15	34	15	54	15	56	25	62
20	39	20	65	20	69	30	70
25	42	25	73	25	79	35	76
30	45	30	80	30	87	40	81
35	48	35	86	35	94	45	86
40	50	40	91	40	100	50	90
45	52						
50	54						
60	57						
70	60						

Table 2.2 Gestational age based on embryonic/foetal characters

Characters	Mean day of earliest appearance (range within parenthesis)
Embryo proper	20.3 (19–24)
Heartbeat	20.9 (19–24)
Allantois	23.2 (22–25)
Spinal cord	29.1 (26–33)
Forelimb buds	29.1 (28–31)
Amnion	29.5 (28–33)
Eye orbit	30.2 (29–33)
Hindlimb buds	31.2 (30–33)
Placentomes	35.2 (33–38)
Split hooves	44.6 (42–49)
Foetal movement	44.8 (42–50)
Ribs	52.8 (51–55)

2.3.5 Foetal Gender Determination

Ultrasonographic gender determination of the bovine foetus was first described by Curran et al. (1989). It was found that the relative location of the genital tubercle (GT), which will become the penis in the male and the clitoris in the female, could be used to determine foetal gender beginning at day 55 of gestation. By this stage, the GT has reached its final location immediately behind the umbilicus in the male or

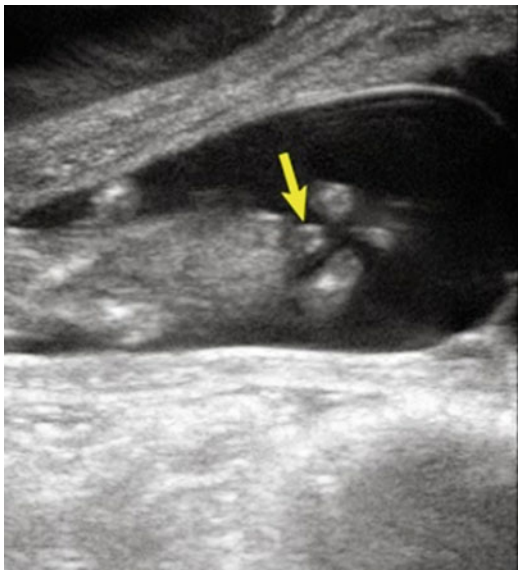


Fig. 2.11 Foetal gender determination based on position of genital tubercle—female foetus

immediately under the tail in the female (Fig. 2.11). Until about 80 days of gestation, the GT usually appears as a highly echogenic lobed structure in both genders.

2.3.6 Diagnosis of Abnormal Pregnancies

Ultrasound investigation addressed many gestational abnormalities like multiple pregnancies (Stroud 1994), early embryonic death (Fissore et al. 1986), and dead foetus (Fissore et al. 1986). It can also be used to monitor the conceptus that are poorly developed for their gestational age and pregnancies in which there is embryonic resorption or foetal abortion. There are, however, a number of ultrasonographic features of normal pregnancies that may mimic disease, and these must be recognized.

Foetal viability can be easily assessed with ultrasound. Foetal heartbeat can be observed clearly from the beginning of about 24 days of gestation. The foetal heartrate (FHR) should be at least 130 bpm in early gestation, increasing to about 190 bpm by 60 days. The amniotic and chorioallantoic fluids should be clear and anechoic until at least 70 days of gestation. After 70 days, the fluids of pregnancy may begin to appear cloudy in normal pregnancies. If the fluid is cloudy before 70 days and there is no detectable heartbeat, embryonic/foetal mortality can be suspected and it is advisable to recheck pregnancies. Separated chorioallantoic membranes may also be seen in cases of late embryonic or early foetal death, often appearing to be floating in the pregnancy fluids (Fig. 2.12).

Dead foetuses often are very heterogeneous, with many areas of high echogenicity that do not correspond to normal bony anatomy. Foetal mummies are

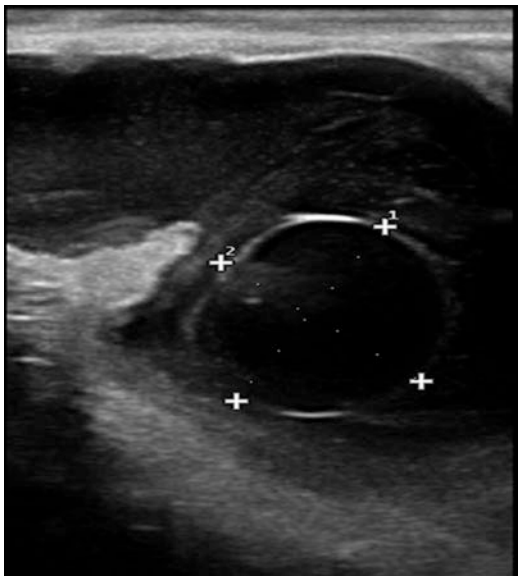


Fig. 2.12 Empty chorioallantoic membrane detected by 80 days pregnancy

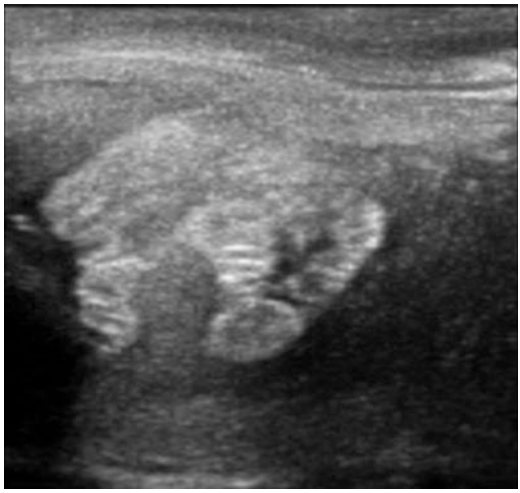


Fig. 2.13 Mummified foetus

easily identified by echogenic bony remnants, nearly complete lack of intrauterine fluid, lack of foetal movement and heartbeat (Fig. 2.13).

There is a lack of well-defined studies to determine the health status of a bovine foetus during the last trimester of pregnancy. The ultrasonographic examination of the late-term pregnancy in cattle consists of collecting pertinent information on the FHR, foetal activity, mean placentome size, thickness of the allanto-amniotic membrane, the uterine adnexa and dam health by transabdominal or transrectal ultrasonography (Buczinski et al. 2007). Transabdominal ultrasonography allows for

diagnosis of abnormal placentomes and hydrallantois. Foetal inactivity or large hyperechoic particles imaged within the foetal annexes are associated with foetal distress or death, and should be reassessed to confirm compromised pregnancy. However, more extensive studies are needed to increase the level of understanding of bovine late pregnancy. Assessment of these parameters should be included in the bovine foetal biophysical profile. The ultimate goal of this knowledge is to decrease neonatal morbidity and mortality in low-and high-risk pregnancies (Buczinski 2009).

2.4 Role of Ultrasonography in Diagnosing Infertile Conditions

The use of ultrasound has proven to be a valuable tool for cattle breeders to assess valuable reproductive information beyond the scope of rectal palpation. Ultrasound investigation addressed many infertility conditions like cystic ovarian disease (Pierson and Ginther 1984), anoestrous (Satheshkumar 2021) and endometritis (Fissore et al. 1986).

2.4.1 Diagnosis of Cystic Degeneration

Cystic ovarian degeneration (follicular and luteal cyst) is an important ovarian dysfunction and a major cause of reproductive failure in dairy cattle with an incidence of 5.2 to 27.9% (Alexandra et al. 2008).

Ovarian follicular cyst (OFC) is a consequence of a mature follicle that fails to ovulate at the appointed time during the oestrous cycle. Earlier, OFCs were defined as fluid-filled structures of $\geq$25 mm in diameter persisting for 10 or more days on the ovarian surface (Roberts 1971). However, with the introduction of ultrasonographic imaging technology, it is determined that follicles of exotic and crossbred cows typically ovulate at 13–17 mm and 10–14 mm diameter, respectively (Ginther et al. 1996; Satheshkumar et al. 2012a, b). So follicles that persist at that diameter or greater may be considered to be cystic. Silvia et al. (2002) defined OFCs as fluid-filled follicle like structures, with a minimum diameter of 17 mm and persisting for more than 6 days in the absence of a CL and clearly interfering with normal ovarian cyclicity. OFCs have a peripheral wall thickness of <3 mm (Fig. 2.14) and are accompanied by a plasma progesterone (P_4) concentration of $\leq$1 ng/ml.

Luteal cysts are also found to be fluid-filled structures but they have a peripheral wall thickness of $\geq$3 mm (Fig. 2.15). Further, the serum P_4 concentration is >1 ng/ml. Ovarian luteal cysts should not be confused with a physiological cystic corpora lutea containing a central fluid-filled cavity.

By the routine rectal palpation, the examiner usually experiences difficulty in differentiating between follicular and luteal cysts. Evaluation of the cysts by ultrasonographic imaging can be used to diagnose between the types of cysts. The

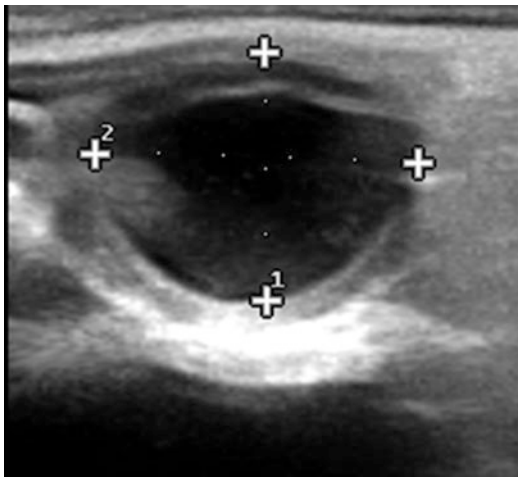


Fig. 2.14 Follicular cyst

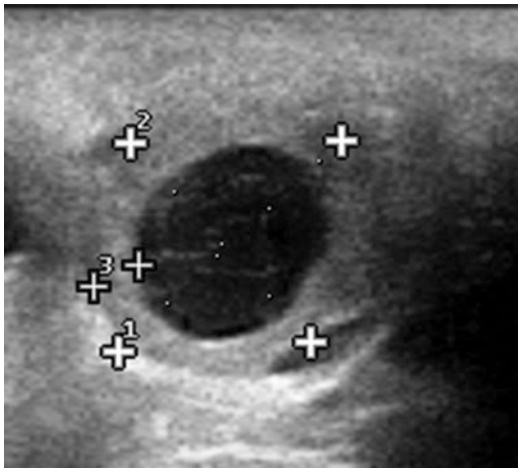


Fig. 2.15 Luteal cyst

following ultrasonographic features of the cysts can be used to differentiate them with precision.

- The persistent anechoic structure of >17 mm diameter with <3 mm wall thickness is considered as follicular cyst.
- The persistent anechoic structure of >17 mm diameter with ≥3 mm wall thickness is considered as luteal cyst.

Web like intra-cystic fibrous strands could be observed in chronic cases of follicular and luteal cysts. This might be attributed to changes in coagulating proteins of the entrapped fluid as reported by Satheshkumar (2021).

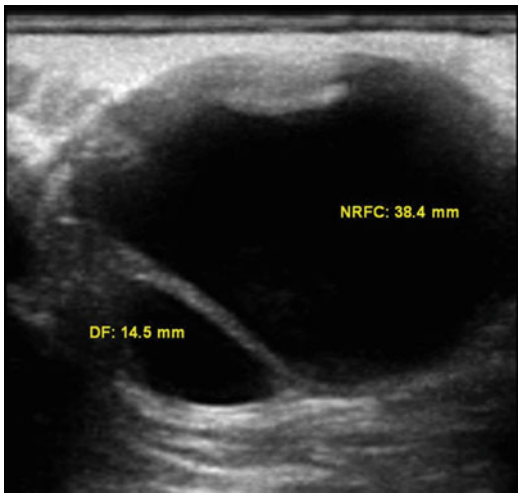


Fig. 2.16 Non-responsive follicular cyst along with the normal dominant follicle

In the recent years, an increase in the incidences of non-steroidogenic large persistent cysts is observed in crossbred cattle. This category of cysts is thin walled, fluid-filled structures uniquely characterized by their uncontrollable acute or chronic increase in diameter and co-existing along with the normal DF and CL (Fig. 2.16). No nymphomaniac or anoestrus symptoms could be observed in the affected animals, instead the cows are cyclic with normal follicular turnover but remains infertile (cyclic non-breeders or repeat breeders). They are extremely non-responsive to regular hormonal therapies and hence referred as 'non-responsive follicular cysts (NRFC)'. Probably, their incidence came to limelight due to the regular application of ultrasonographic diagnostics. These non-responsive cysts are inert structures, and the only remedy is their mechanical evacuation. Ultrasound guided trans-vaginal or trans-gluteal route of follicular cyst evacuation was successfully reported in crossbred cows (Satheshkumar 2018a, b; Satheshkumar et al. 2018). Following cystic evacuation, animals are subjected for programmed breeding protocol, and conception rate of 66.7% was recorded in affected animals (unpublished data).

Thus, ultrasonography proved to be a part of therapeutic approach alongside its inevitable role in diagnostics.

2.4.2 Anoestrum

Postpartum anoestrus (PPA) is an ovarian dysfunction which delays the resumption of cyclicity after parturition and is attributed to many factors ranging from negative energy balance to stress of any origin (Opsomer et al. 1996; Kumar et al. 2014). Ovaries of acyclic cattle were often referred to be inactive (Roberts 1971). However, intervention with ultrasonography revealed that acyclic animals also displayed variable degree of ovarian activity, characterized by follicular turnover but without expression of oestrus signs and ovulation (Satheshkumar 2021).

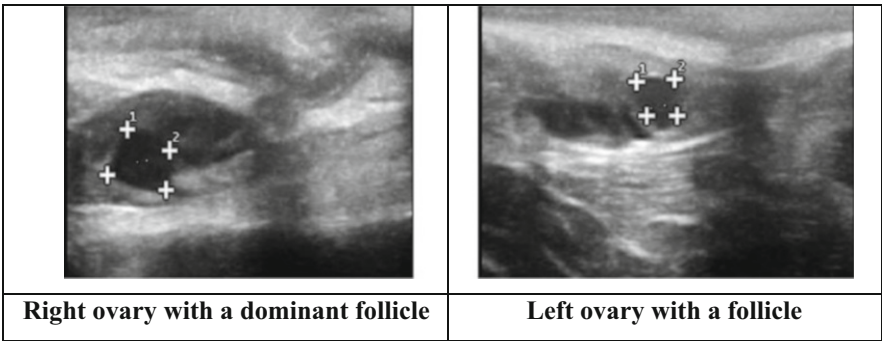


Fig. 2.17 Normal follicular turnover in both the ovaries of acyclic cows. (**a**) Right ovary with a dominant follicle, (**b**) Left ovary with a follicle

In acyclic animals, both the ovaries exhibit extensive follicular activity, and the DFs develop in a wave-like fashion similar to that of normally cycling animals. A maximum of two waves occur within a span of 25 days, with each wave extending for a period of 10–14 days. The follicular wave exhibits coordinated growth, static and regression phases as that of cycling cows. The DFs reach a diameter of >10 mm on par with the size of a normal ovulatory follicle (Fig. 2.17) and remain static for a prolonged period before entering the regression phase. The deficient metabolic and endocrine status of the follicular microenvironment would have affected the steroidogenic capacity of the follicular cells and the oestrogen production thereon. Development of oestrogenically inactive large follicles and lack of ovulation are found to be the major factors for the incidence of PPA in crossbred cows. Failure of the intra-follicular IGF/IGFBP system might prevent the follicular maturation and hinder the normal cascade of events towards ovulation leading to non-expression of oestrus signs and anovulation in acyclic animals (Satheshkumar et al. 2019a). Thus, uninterrupted follicular growth and atresia of follicular entities without culminating in ovulation are documented with the application of ultrasonography.

2.4.3 Endometritis

Endometritis is a common condition affecting dairy cattle, and it negatively affects reproductive performance. Clinical endometritis is characterized by the presence of purulent or muco-purulent uterine discharge (Sheldon et al. 2006), and about 15% of dairy cows have clinical signs of such uterine disease that persist beyond 3 weeks postpartum. Subclinical cases of endometritis are the inflammation of the uterine endometrium without any overt expression of clinical signs and characterized by clear oestrual discharge. Nearly 30–35% of dairy cows are affected by this subclinical infection between 4 and 9 weeks postpartum. (LeBlanc 2008) and thus fertility is negatively affected. Transrectal ultrasonography may be used to evaluate cows for signs of endometritis. On ultrasound investigation, accumulation of intrauterine fluid

containing echogenic particles and thickening of the endometrium due to endometrial oedema and inflammation are considered to be the indicators of endometritis. However, ultrasonography alone does not always provide a definitive diagnosis of endometritis.

2.4.4 Diagnosing Congenital or Acquired Anomalies

Diagnosis of congenital anomalies in heifers is a determining factor to decide upon its breeding capability in the earlier ages. Even though anoestrus associated with utero-ovarian agenesis/aplasia could be detected by routine per-rectal examination, conditions like persistent hymen can be noticed only when the animal becomes cyclic. Massive accumulation of secretory fluid within the uterine lumen and vaginal canal is the alarming features in such anomalies (Fig. 2.18) which requires immediate intervention.

Congenital or acquired peri-ovarian anomalies and their effect on the mammalian fertility is a subject of interest for many decades due to its scientific and clinical importance. It is proved beyond doubt that ultrasonographic investigation is the best option for diagnosing such anomalies of delicate peri-ovarian structures. Physical and physiological relationship between ovary, bursa and oviduct plays a major role in gamete capture, transport and fertilization. Compromises in ovario-bursal and oviductal structures due to inflammation and adhesions lead to reproductive failure ranging from temporary infertility to permanent sterility. Severe ovario-bursal adhesions affect the ovulation process by masking the ovarian surface. Similarly occlusion of fallopian tube due to inflammation or fibrosis leads to fertilization failure or tubal entrapment of fertilized embryos (Shivhare et al. 2012). Animals with bilateral ovario-burso-tubal adhesions are sterile and their diagnosis in the earlier stages is warranted (Satheshkumar et al. 2019b). Hyperechoic borders of

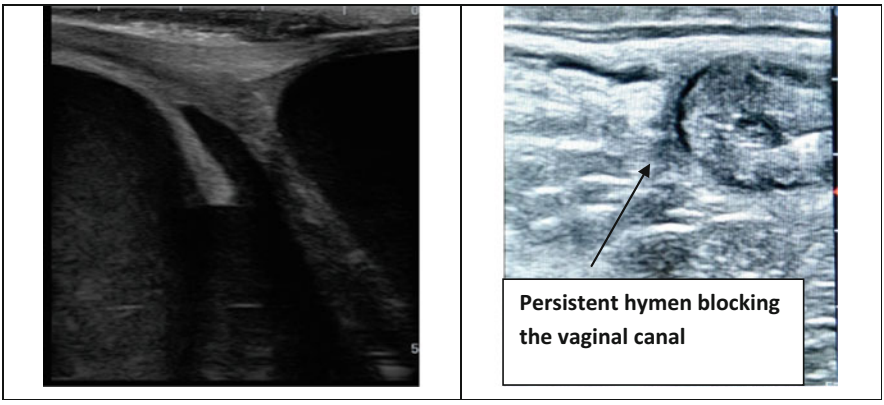


Fig. 2.18 Ultrasonographic imaging of uterus and cervix with accumulated mucus due to blockage of vaginal passage

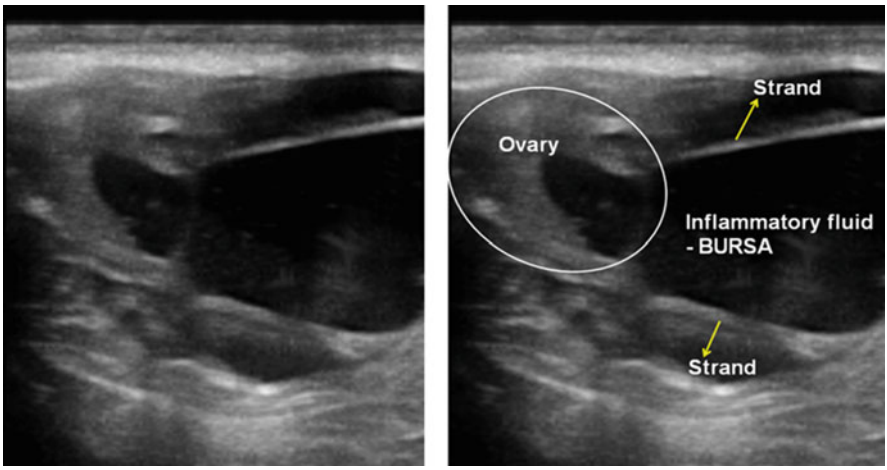


Fig. 2.19 'Cystic ovarian bursa' encapsulating the ovary

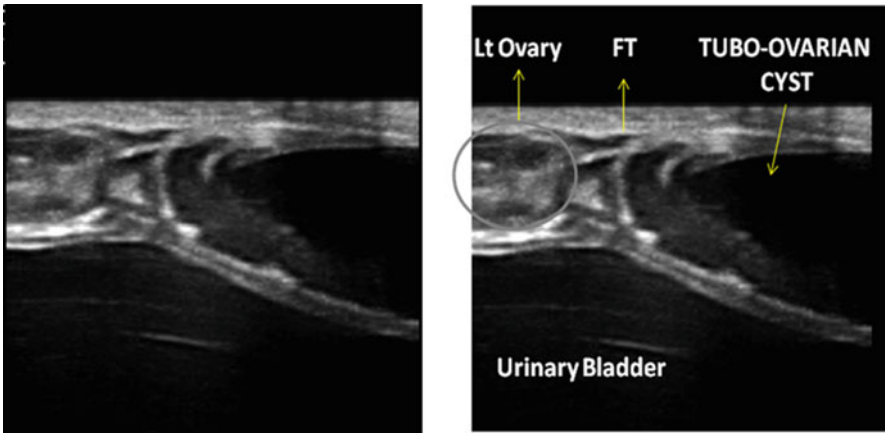


Fig. 2.20 'Tubo ovarian cyst' with fluid accumulation in fallopian tube

the ovario-burso-tubal structures surrounded by anechoic fluid accumulation indicate the severity of inflammation (Figs. 2.19, 2.20, 2.21).

Ultrasonographic investigation provides the actual degree of affection and the fate of future reproductive capability of that particular animal.

2.5 Utero-Ovarian Haemodynamics and Doppler Imaging

The blood supply to any organ is closely related to the function of that organ and reproductive tract is no exception. Consequently, assessment of blood flow characteristics is an important evaluation parameter of reproductive function. Colour

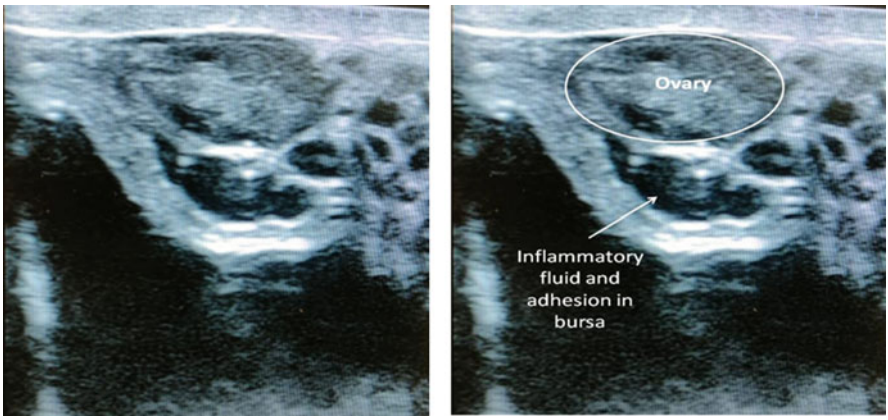


Fig. 2.21 'Ovario-bursal' adhesion

Doppler imaging ultrasonography is routinely used in human gynaecology and obstetrics as the diagnostic gold standard. In the recent years, the use of Doppler imaging is gaining momentum in veterinary practice also. Colour Doppler imaging provides important information about the physiological/pathological status of follicles and CL supporting clinical diagnosis and therapeutic decisions. Several studies have demonstrated the relationship of blood flow and utero-ovarian function throughout the oestrous cycle and pregnancy (Acosta et al. 2002, 2003; Miyamoto et al. 2006; Herzog et al. 2010; Satheshkumar et al. 2012b; Satheshkumar 2018b).

2.5.1 Basic Principles of Doppler Ultrasonography

Qualitative assessment of blood flow in capillary networks draining reproductive organs can be carried out non-invasively by colour Doppler imaging ultrasonography, and the quantitative parameters of blood flow can be assessed by pulsed/spectral Doppler ultrasonography (Ginther 2007a, b). Blood flow to the organ is displayed in colour superimposed on a two-dimensional, grey-scale ultrasonographic image (colour Doppler) or as a graph depicting pulsed Doppler-spectral analysis of blood velocity in a small area of a large blood vessel (spectral Doppler).

Transducer should be placed as close as possible to the ovary, and colour blood flow mapping of the ovarian structures is conducted in various transverse sections. In the ultrasound equipment, activation of the colour Doppler mode enables the examiner to identify the blood flow as coloured signals on the ovaries, tubular genitalia and the blood vessels in the adjacent region like middle uterine arteries, ovarian arteries, etc. The colour mode determined the direction of blood flow and the blood flow area within the structure. Basically, the Doppler signals are viewed in a range from red to blue colour. The variations in colour signals do not represent the arteries and veins, but it represents the direction of blood flow (Fig. 2.22).

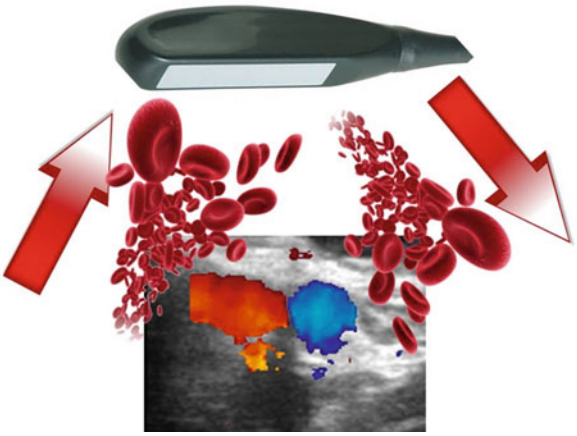


Fig. 2.22 Colour Doppler signals according to the direction of blood flow

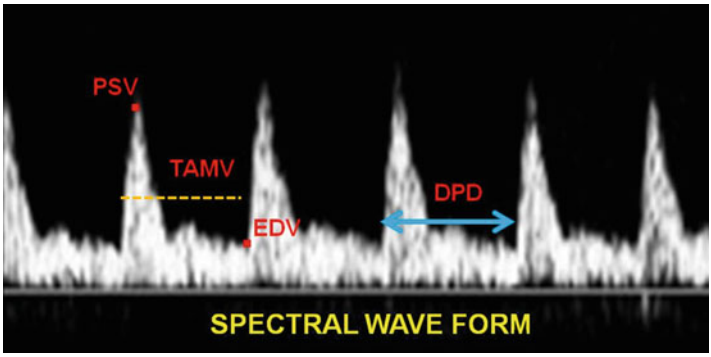


Fig. 2.23 Spectral waveform and various velocimetric indices

- When the blood flow towards the probe, it appears as red to orange in colour.
- When the blood flows away from the probe, it appears as blue in colour.

Since the uterine (mesometrial, endometrial and myometrial) and ovarian (mesovarium, luteal and follicular) arteries have a tortuous anatomy, blood flow usually appears as red and blue colour adjacent to each other.

The arteries and veins can be differentiated based on spectral Doppler study. Normally, the arteries will be evincing pulsations but the veins are devoid of pulsation.

Switching over to pulsed wave spectral Doppler mode, the Doppler gate is positioned over the blood vessel and a spectral mode waveform is taken for calculating an array of velocimetric indices recorded during the systolic and diastolic stages of the cardiac cycle. In the spectral mode, blood flow velocity variations are represented as a graphic wave form called spectrum (Fig. 2.23). By convection, waveforms above and under the baseline indicate the red blood cells moving toward

and away from the transducer, respectively. The spectrum provides peak systolic (PSV), end diastolic (EDV), and time-average maximum (TAMV) velocities in m / sec or cm/sec (Ferreira et al. 2011). Doppler pulse duration (DPD) can be recorded by measuring the linear distance between two subsequent EDV points and is expressed in milli seconds—ms (Satheshkumar et al. 2013; Satheshkumar 2018b).

- PSV is the maximum point along the length of the spectrum.
- EDV is the ending point of the cardiac cycle.
- TAMV is the average of spectral maximum blood flow velocities.
- DPD is the duration of a cardiac cycle and is indirectly proportional to the pulse frequency.

Apart from these velocity and duration indices, Doppler device automatically calculates pulsatility index (PI) and resistance index (RI) based on inbuilt formulation. These indices have negative correlation with the vascular perfusion of the tissue downstream from the sample gate and indicate the resistance to the blood flow. A PI and RI value of <1 usually reflects the decreased resistance and free flow of the blood supply to any organ**.**

Intensity of the follicular and luteal blood flow is graded by visual evaluation of the proportion of the peri-follicular and CL area filled with colour Doppler signals and is subjectively expressed as low, medium, high and very high (Kaya et al. 2017).

The knowledge of ovarian vascular development is of fundamental importance to understand the processes that ensure the reproductive success. Continued re-modelling of the ovarian vascular network supports the cyclic development and regression of follicular and luteal structures throughout the reproductive life. The uterine branch of the ovarian artery is prominent and forms a utero-ovarian arterial anastomosis between the uterine and ovarian arterial systems. Severance of the utero-ovarian arterial anastomosis on the CL side during the oestrous cycle interferes with ovarian function more than severance of the ovarian artery (OA) indicating the importance of the anastomosis to CL blood flow. Further, the ovarian angiogenesis or neovascularisation is a complex process in which a delicate balance between promoters and inhibitors is maintained. Disturbance of this balance may result in a disrupted physiologic state or various pathologic conditions.

2.5.2 Follicular Vascularity

Follicles require coordinated vascular development and adaptations as their development advances towards ovulation (Martelli et al. 2017). Active angiogenesis or neovascularization of follicles provides important signalling for the development and steroidogenic functions (Robinson et al. 2009). Follicular angiogenesis and its related outcome on dominance, atresia and ovulation were recorded in exotic (*B. taurus*) cows and heifers (Acosta 2007; Siddiqui et al. 2009; de Tarso et al. 2017) and in crossbred cows (Satheshkumar et al. 2013; Satheshkumar 2018b).

2.6 Peri-Follicular Blood Flow and Its Effect on Ovulation

Blood supply to individual follicle is critical for allowing it to acquire ovulatory capacity (Acosta et al. 2003). Regulatory role of vasoactive substances released during luteinizing hormone (LH) stimulation had an important role in the ovulation process. Subsequent reports also related the importance of pre-ovulatory follicular vascularity to the conception rate and fertility (Siddiqui et al. 2009; Varughese et al. 2013).

2.6.1 Intensity of Blood Flow

The sequential follow up of peri-follicular Doppler signals indicating the intensity of blood flow revealed that the ovulatory follicle (OF) was well vascularized with consistent blood flow signals surrounding the base of the follicular antrum, while the adjacent subordinate or atretic follicles were devoid of detectable blood flow (Fig. 2.24a). The intensity of peri-follicular blood flow increased as the time of ovulation approached which was evident by enriched colour Doppler signals covering over 70% of the OF circumference (Fig. 2.24b). The increased intensity of peri-follicular Doppler signals indicated that there was an active neovascularization process and increasing arteriolar network encasing the OFs (Ginther 2007a, b). The supply of hormones, growth factors, nutrients and oxygen is ensured as a result of having an adequate blood supply to the follicles. Thus, it is confirmed that there is a strong association between the angiogenic/neovascularization process and the selection of the OF during the final phases of development in cattle (Varughese et al. 2017; Satheshkumar 2018b).

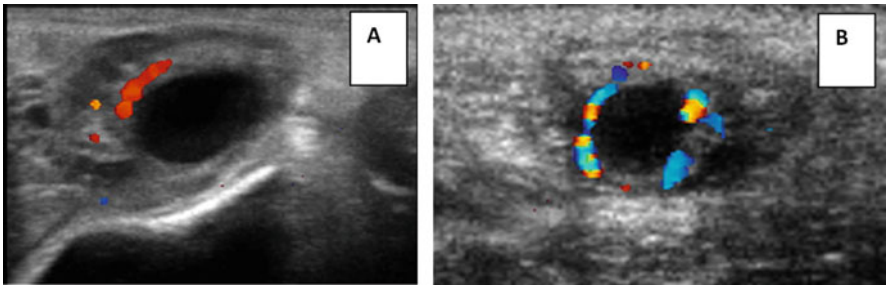


Fig. 2.24 (**a**) Pre-ovulatory follicle with peri-follicular blood flow signals at the base of the follicle in the initial stages (Note: subordinate follicles without detectable signals) and (**b**) Doppler signals covering over 70% of follicular circumference

2.6.2 Blood Flow Parameters in Relation to Ovulation

The blood flow not only determines the selection of the OF but it also have a definitive role on the ovulation. Blood flow velocity parameters and indices of the peri-follicular blood flow of the OF were studied from the day of induced oestrus (Day 0) until the time of ovulation. The follicular size and the circumferential blood flow area gradually increased until the time of ovulation (Palmer et al. 2006).

As mentioned earlier, ovulation is delayed in 45.4% of oestrous cycles in crossbred cows. Interpretation of sequential changes revealed that the mean DPD (874.3 ± 57.0 ms) and PI (0.62 ± 0.01) values were less on the onset of oestrus (Day 0) in the animals that ovulated normally (Fig. 2.25a) when compared to the animals which ovulated late (1140.6 ± 27.5 ms and 1.28 ± 0.15, respectively) (Fig. 2.25b). The decreased DPD and PI values in the former category are indicative of a relatively rapid pulse frequency and greater vasodilation and vascular perfusion (Anteby et al. 1996; Palmer et al. 2006; Siddiqui et al. 2009). Scanning electron microscopic observations of ovarian corrosion casts by Macchiarelli et al. (2006) confirmed the fact that capillaries of pre-ovulatory follicles are dilated to a greater extent than that of earlier developing follicles. Based on ROC analysis, it is determined that 929 ms

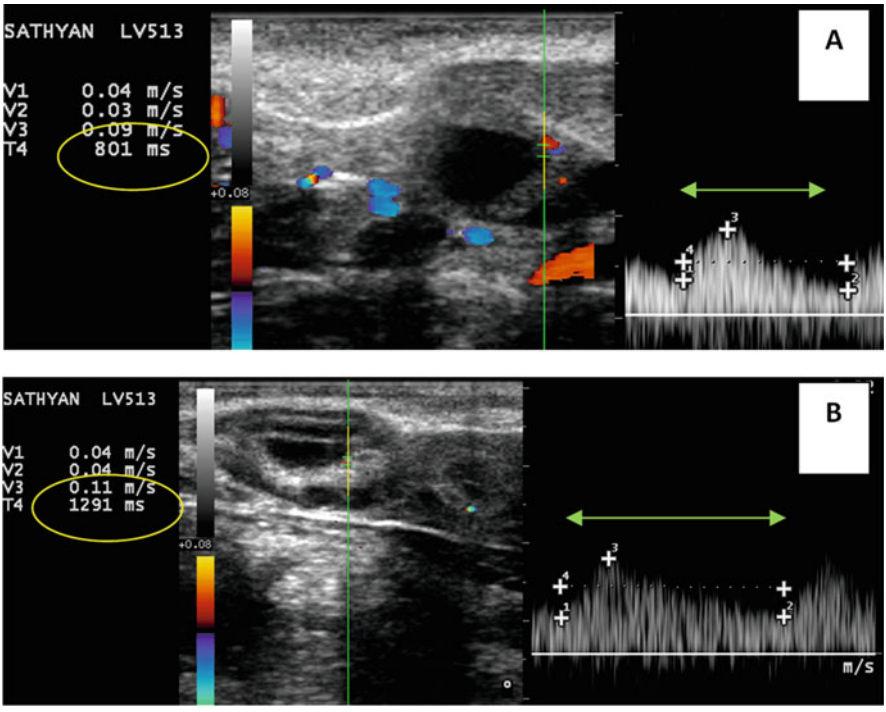


Fig. 2.25 Measurement of Doppler pulse duration (DPD): (**a**) Normal ovulatory follicle with rapid pulse frequency and (**b**) Follicle of delayed ovulation with slow pulse frequency

is the optimal cut-off point for DPD and values lesser than this point indicates the normal ovulatory status and vice versa with higher values (Satheshkumar 2018b).

It is confirmed that the cascade of events induced by the LH surge in pre-ovulatory follicles was closely associated with a local increase in the peri-follicular blood flow, a functional adaptation that is important for the impending ovulation to occur.

- The lesser DPD (<929 ms) and PI (<1) values on the day of oestrus are positive indicators of normal ovulation process and any deviations in these blood flow parameters delayed the timing of ovulation.

This finding is a valuable diagnostic parameter and is an important consideration for future studies involving regulation of follicular vasodilation to hasten the ovulation process in cattle.

2.7 Luteal Vascularity

The CL is a site of intense angiogenesis and is the most vascularized structure in the body (Acosta et al. 2002). The luteal vascularity enables the luteal cells to obtain the nutrient substrates and hormone precursors that are indispensable to support P_4 biosynthesis and also to release the synthesized steroid into the general circulation (Miyazaki et al. 1998; Fraser and Wulff 2003). The nature of vascularity reflected the functional status and steroidogenic capacity of CL (Herzog et al. 2010; Kaya et al. 2017).

2.7.1 Luteal Vascularity During Normal Cycle

In a sequential study luteal haemodynamics throughout the oestrous cycle from the onset of oestrus (Day 0), blood flow could be observed as less intense sparse colour Doppler signals around the periphery of the luteal tissue from Day 3, which increased gradually and covered more than 70% of the luteal circumference during the mid-luteal phase (Day 9–10). The progressive increase in luteal blood flow could be attributed to the increasing endocrine activity of the structure. Herzog et al. (2010) and Kaya et al. (2017) recorded a significant correlation between the proportion of luteal blood flow and P_4 secretion. The levels of VEGF and FGF, the pro-angiogenic factors, which are involved in neovascularisation after ovulation are found to be elevated during luteinization and throughout the development of the CL during mid-luteal phase, confirming the role of luteal vascularity (Zalman et al. 2012). An intense increase in blood flow is appreciated between Day 15 and 17 of the oestrous cycle, which can be appreciated by the penetration of colour Doppler signals deep into the luteal parenchyma. This unique feature is correlated to the pulsatile release of prostaglandin (PG) from the uterus as indicated by drastic increase in the plasma PGFM concentration during the period (Miyamoto et al. 2006). After that period, the

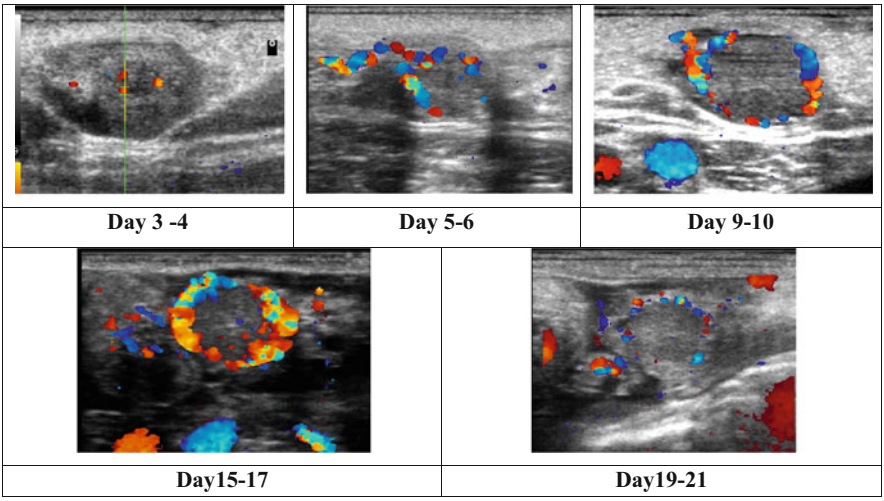


Fig. 2.26 Sequential changes in luteal blood flow during normal oestrous cycle (Day 3–4), Day 5–6, Day 9–10, Day15–17, Day19–21

blood flow signals decrease and found to be restricted to the periphery as the CL, which started regressing towards the final stages of the cycle (Day 19–21). Thus, the increased vascularization of CL during Day 15–17 of the cycle was the first indication of initiation of luteolytic phenomenon (Fig. 2.26).

Programmed induction of luteolysis is possible by administration of exogenous PG during the mid-luteal phase, but the early CL (till Day 4 after oestrus) is refractory to PG treatment (Beal et al. 1980). The non-responsiveness of early CL was suggested to be due to luteal insensitivity or insufficient numbers of PG receptors (Duchens et al. 1994). PG exerts its luteolytic effects by binding to plasma membrane receptors (Sakamoto et al. 1994) and apoptosis of luteal cells is caused by reduction in blood flow and hypoxia induced inhibition of steroidogenic enzymes. Although these events contribute to the later stages of luteolysis, little is known about the cascade of physiological events initiating the luteolytic process. Colour Doppler imaging has given a solution for this gap of information. Acosta et al. (2002) and Shrestha and Ginther (2011) observed an acute increase in luteal blood flow in response to a conventional dose of PG administered in both the early-luteal phase (Day 3) and the mid-luteal phase (Day10). However, Satheshkumar et al. (2014b) documented that the increase in vascularity in response to exogenous PG was restricted only to the periphery of the CL in the early-luteal phase group, while the deeply radiating property of the vascular perfusion was recorded in mid-luteal phase group. Investigations on luteal blood vessels by Shirasuna et al. (2008) revealed that microcapillary vessels were present in both the peripheral and central regions of early and mid-luteal phase CL, but large blood vessels (>20 μm) were abundant in the periphery of the mid CL when compared to the early CL. Miyamoto and Shirasuna (2009) suggested that PG stimulated endothelial Nitric Oxide

Synthase—Nitric oxide (eNOS-NO) system caused the vasodilation of peripheral blood vessels of the mid CL. The eNOS-NO system would have dilated the microvascular bed within the mid CL, resulting in free flow of blood deep into the luteal parenchyma. This vascular perfusion would have enabled the luteolysin to reach the deeper luteal cells and initiated the luteolytic process in mature mid-CL, rather than the immature early CL. This salient difference in the vascularity between the two stages of development might be the determining factor that initiates the lysis in response to exogenous PG in crossbred cattle. In natural cycle, gradual increase in blood flow could be observed for an extended period of 2–3 days before spontaneous luteolysis due to the pulsatile release of PGF at sequential intervals (Ginther 2007a, b), but in PG treated cycles the vascular perfusion episode was acute.

2.7.2 Luteal Vascularity During Pregnancy

A functional CL is required for establishment of pregnancy because P_4 supports pre-implantation embryonic development enabling maternal recognition of pregnancy of farm animals (Grana-Baumgartner et al. 2020). Late embryonic mortality usually occurs between 18 and 42 days of gestation and contributed for 3.2–42.7% of pregnancy losses in cattle (Santos et al. 2004; Pohler et al. 2016). Hence, the endocrine competence of CL is very crucial during this period.

Visual analyses of luteal vascularity during the first trimester of pregnancy revealed that the intensity of blood flow was very high covering more than 90% of the luteal circumference and even reaching the deeper areas of luteal parenchyma between 25 and, 35 days of gestation and the high intensity is maintained with the advancement of pregnancy (Fig. 2.27). This high intensity of luteal blood flow in the early stage of pregnancy is suggestive of high degree angiogenesis and the increased blood supply from the ipsilateral uterine artery and ovarian artery (Pinaffi et al. 2018). The high intensity of luteal blood flow during the early stage of gestation might be a supportive mechanism for synthesizing and releasing optimum levels of P_4 in order to sustain the pregnancy. It could be inferred that active neovascularization, expanded luteal vascular bed and increased pulse frequency of

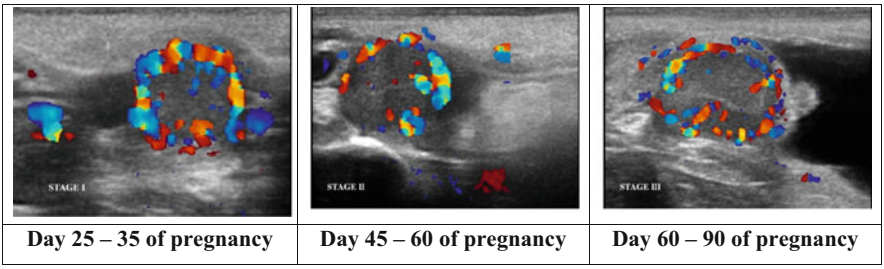


Fig. 2.27 Intensity of luteal blood flow during various stages of early pregnancy, Day 25–35 of pregnancy, Day 45–60 of pregnancy, Day 60–90 of pregnancy

luteal blood flow during the first trimester of pregnancy allowed adequate blood supply to the CL and thus supported the endocrine function and early embryonic/foetal survival.

Blood flow has been suggested to be more appropriate than size for CL function evaluation (Herzog et al. 2010) because CL vascularization plays a key role in regulating luteal function (Miyamoto et al. 2006). Evaluations of CLBF could, consequently, be useful to detect pregnancy failures or to predict pregnancy rates after embryo transfer or timed-AI.

2.8 Utero-Ovarian Haemodynamics in Disturbed Fertility

Colour Doppler imaging facilities have provided new insights into causative factors of the common fertility disorders in cattle. An altered blood supply to the uterus and ovaries could disturb the normal cycle and conception in cattle.

2.8.1 Blood Flow Parameters in Relation to Anoestrum

Blood flow in OA is related to the vascularity of intra-ovarian arteries and the ovarian follicles. The PI, RI and DPD indices of the OA ipsilateral to the ovary having the largest follicle were higher in anoestrus than in oestrus group of animals (Satheshkumar et al. 2017). Thus, increased flow resistance (indicated by high PI and RI) and slow pulsation (indicated by high DPD) of the blood flow in OA of anoestrus animals are the major factors affecting the vascular perfusion of follicles. Thus, altered blood flow parameters of OA are proved to be a predisposing factor for deficient follicular development and maturation in anoestrus cattle.

2.8.2 Blood Flow Parameters in Relation to Cystic Degeneration

Díaz et al. (2019) recorded marked changes in blood irrigation area of walls of persistent follicles simulating the early stages of development of follicular cyst. They found that the Doppler signals of blood flow were less in walls of persistent follicles than in normal DFs (Fig. 2.28a). As described earlier, there will be variation in the wall thickness between the follicular and luteal cyst. The Doppler technology shows the blood flow in the wall, and the area of the blood flow therefore gives a clearer view of the thickness of the wall (Fig. 2.28b).

2.9 Conclusion

Growth and regression of ovarian structures in cattle can be monitored with ultrasound on a sequential basis and in a non-invasive manner. Ultrasound is more accurate than rectal examination in determining the dimensions of ovarian structures.

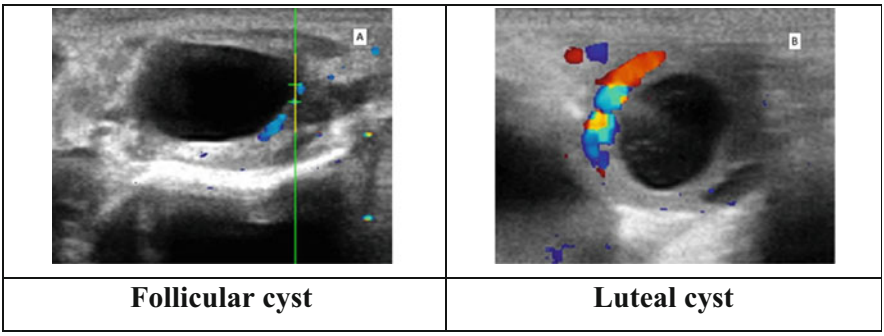


Fig. 2.28 Vascular pattern in follicular and luteal cysts, (**a**) Follicular cyst, (**b**) Luteal cyst

Thus, the application of real-time ultrasonography for monitoring ovarian function in cattle has advanced the understanding of follicular and luteal dynamics and its regulation. Colour Doppler sonographic studies showed that it is a useful technique for the investigation of the utero-ovarian blood flow and provide new information about physiological changes during various phases of cycle. Based on the basic studies, it could be concluded that the control of angiogenic development in the ovary could be a useful tool to improve animal reproductive performances. In addition to manual per-rectal examination, ultrasonography should be included in the infertility investigation to examine and evaluate the reproductive status of cattle. This enables to assess the architecture of the ovaries, uterus, reproductive vasculature and surrounding structures for accurate diagnosis of the aberrations. Early diagnosis with appropriate therapeutic intervention will aid in improving the bovine fertility and production thereof.

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Infrared Thermal Imaging and Its Application in Animal Reproduction

3

S. Jeyakumar, A. Kumaresan, Mukund A. Kataktalware, A. Manimaran, and K. P. Ramesha

Abstract

Infrared thermography (IRT) is a non-invasive technique that measures mid- to long-wave infrared radiation emitted from all objects and converts this to temperature. The thermogram depicts visual representation of the energy emitted, transmitted, and reflected by an object and its environment. IRT is a simple, effective, on-site, and non-invasive method that absorbs surface temperature, which is emitted as infrared radiation and generates a pictorial image without causing radiation exposure. The most direct use of the thermal imaging camera is for measuring animals body surface temperature, and as a tool for ascertaining changes in body temperature associated with physiological and pathological status of the organ or body surface areas. IRT has potential application in the field of animal reproduction especially monitoring reproductive events, viz. estrous cycle, pregnancy. In females, cutaneous temperature of vulvar and perivulvar areas may be useful for increasing the awareness about the ovulation time. In vulvar areas, the thermal radiation is related to the cutaneous or subcutaneous blood flux variations which is mainly due to change in the circulating levels of estrogen from growing follicles/pre-ovulatory follicle. In males, IRT is considered as a most promising non-invasive and non-contact imaging technique to study scrotal thermoregulation and infertility problems associated with testicular pathologies and thermal stress. IRT is also recommended as a non-invasive tool in the evaluation of bulls during breeding soundness examination. However, thermography can be routinely applied in animal reproduction as a robust technique only after establishing a species and/or breed specific thermal profile associated

S. Jeyakumar (✉) · M. A. Kataktalware · A. Manimaran · K. P. Ramesha
Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

A. Kumaresan
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

A. Kumaresan, A. K. Srivastava (eds.), *Frontier Technologies in Bovine Reproduction*, https://doi.org/10.1007/978-981-19-3072-0_3

with physio-pathological events of interest in reproduction and would pave a way for development of temperature associated tool or technology for remote monitoring of reproductive events.

Keywords

Infrared thermography · Applications · Bovine · Reproduction

3.1 History and Applications of Infrared Thermography

The discovery of infrared radiation goes with the name Sir Frederick William Herschel (1738–1822) a great astronomer and space surveyor, who demonstrated that there was an increase in temperature of refracted light from sun using a prism and a thermometer (Fig. 3.1. Image source: http://coolcosmos.ipac.caltech.edu/

Fig. 3.1 Sir Frederick William Herschel

cosmic_classroom/classroom_activities/hershel_bio.html). He was surprised to observe rise in temperature beyond the red part of the spectrum due to presence of an "invisible light" and called them "Calorific Rays" and the term "infrared" appeared in the late nineteenth century. John Herschel, his son in the year 1840 created first heat picture, the so-called thermogram and the crude image produced on a paper called as "thermograph." During the Second World War, the Germany developed first electronic infrared camera for use on tanks for night vision. (Ricca 2013). Later, in the nineteenth century, Samuel Langley made improvements in IR sensing and the sense of heat could be identified from long distance of 400 m. Around the first half of the twentieth century, by using converters and photon detectors many improvements were made in IR detection for army applications and in the year 1950s, the IR camera found a wider application in the field of science and industry. In 1970s, portable IR scanning devices with television systems with battery backup were produced. Liquid nitrogen was used as a cooling agent in these devices which limited some of its applications and in 1980s first thermo-electrically cooled systems having more portability were produced. By 1990s, hand-held non-cooled camcorder was produced. After 2000, technology of thermal imaging has developed rapidly, where low-cost compact devices and automated systems are available in market (FLIR systems 2004). Infrared wavelength energy is converted into visible light display by thermal imaging cameras. The color of warmer objects is displayed distinctly from their surroundings; warm-blooded animals easily become differentiated from their environment, both at day and nighttime. On thermogram, the warmest regions appear white and the coolest region appear either blue or black (Colak et al. 2008 and http://coolcosmos.ipac.caltech.edu/cosmic_classroom/classroom_activities/herschel_bio.html) and in the recent times application of various thermal image processing software gives advanced color palates to differentiate thermal profile of a thermogram and image analysis tools and application for on-spot and remote communication of interpretation.

3.2 Medical Infrared Thermography

Earliest medical application of infrared thermography was to diagnose early stages of breast cancer since infrared cameras could visualize a hot spot in the thermal image due to localized rise in temperature indicating highly vascularized tumor cells/mass. Since then, the applications of infrared thermal imaging technology occupied an inevitable space in medical application. Medical infrared thermography is a non-radiating and contactless technology to monitor physiological functions related to skin temperature control. Medical thermography was born in 1957 when a surgeon, Dr. R. Lawson discovered that his breast cancer patients had higher skin temperature over the cancer area. Since the 1970s, thermography has been used in many areas of medicine and used as an auxiliary tool for disease diagnosis based on thermal changes in skin surface temperature around the area of interest (Mikulska 2006; Hildebrandt and Ammer 2010). Human skin emits about 80% of infrared radiation in the wavelength range of 8–15 μm (Steketee 1973). Infrared

thermography (IRT) in medicine is used as a non-invasive tool for diagnosis of pathophysiological functions, which are linked to surface temperature of skin. There are various applications of medical infrared thermography in the field of human medicine, viz. skin temperature differences between paired limbs (Sherman et al. 1996), diabetic neuropathy (Ring 2004) and vascular disorders (Bagavathiappan et al. 2009), thermoregulation studies (Ludwig et al. 2014; Formenti et al. 2013), dermatology, rheumatologic diseases and bowel ischemia (Brooks et al. 2000). There are several applications of medical infrared thermography in the field of human medicine, such as open-heart surgery, neurological disorders, vascular diseases, reflex sympathetic dystrophy syndrome, urology problems, diabetes, and mass fever screening. Infrared thermography has become a routine screening and evaluation method for diagnosis and to monitor therapeutic response of various treatment protocols/drugs to breast cancer (Hildebrandt and Ammer 2010) in western countries. In recent times, IRT is applied as a diagnostic tool in human medicine in the field of oncology, allergic diseases, angiology, plastic surgery, rheumatology, ophthalmology, and dentistry.

3.3 Application of Infrared Thermography (IRT) in Veterinary Medicine

3.3.1 Body Temperature Measurement

Mammals and birds maintain their body temperature in homeothermy through the process of thermoregulation. Body temperature and its variations are considered as one of the most important physiological parameters to assess health status, well-being (stress) of animals and birds. Nutrition, production, reproduction, endocrine and metabolic status, physiological activity, and stress responses are generally linked to thermoregulatory mechanism and it is reflected through body temperature of an animal/bird. Body temperature of cattle is measured at various anatomical locations including rectum, ear (tympanic), vagina, reticulum-rumen, and udder (milk) (Yoshioka et al. 2019; accessed on-line: http://ap.fftc.agnet.org/files/ap_policy/1067/1067_1.pdf). Core body temperature, environmental conditions, and peripheral blood circulation influence the skin surface temperature /peripheral temperature of the particular region of the body. Circadian rhythms have influence over body temperature and are linked to environmental parameters and to a given thermoneutral conditions (Lowe et al. 2001; Piccione et al. 2005).

Body core temperature is measured at different locations such as rectal, tympanic, and vaginal by veterinarians to assess health or disease status of an animal. In general, rectal thermometer is used commonly to measure the animal's body core temperature. However, animal needs to be restraint every time for single/continuous measurement of body temperature when we use rectal thermometer and this may result in variations due to handling stress and positioning of thermometer. There is contact, contactless, and remote monitoring methods available for measuring core body and surface temperature of animals and birds. Various temperature

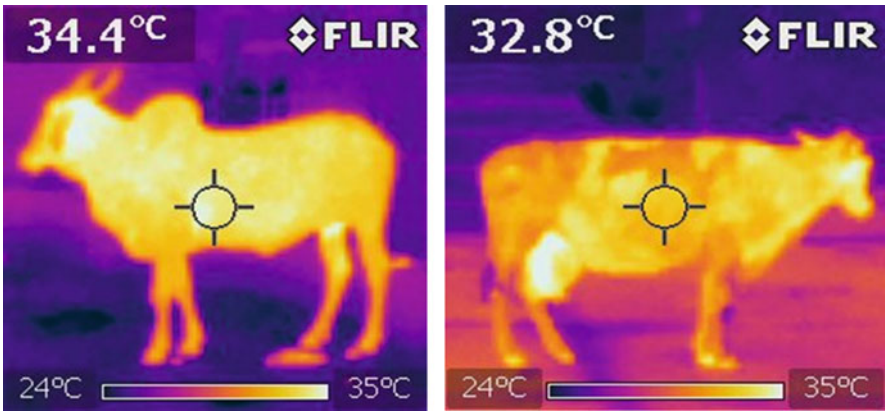

Fig. 3.2 Infrared thermogram showing body surface temperature pattern in Deoni (*Bos indicus*) and HF crossbred (*Bos indicus* x *Bos taurus*) cattle

measurement devices like thermometry (rectal, oral, axillary, esophageal, etc.), implantable data loggers, implantable and skin surface radio transmitters, gastrointestinal devices (radio-telemetry pills or miniaturized dataloggers), non-surgical implants (rectal, aural, and vaginal), Passive Integrated Transponder (PIT) tags (implantable temperature sensitive transponders), infrared thermometry (IR thermometer), infrared thermography (thermal imaging), and temperature sensitive paints are available and used according to the need, subject, and duration of observation.

All the surfaces with temperature above 0 K emit infrared radiation within the wavelength range of 7–14 micrometer this is referred to as infrared radiation. Infrared radiation is measured using a specialized thermal imaging camera, and the technique is referred as Infrared Thermography (IRT) (McCafferty 2013). The heat loss in animals' ranges between 40 and 60%, and this is within the wavelength range of infrared radiation (Stewart et al. 2005). Thermography has advantage over other methods that it is a non-invasive, safe technique which allows for a contactless assessment of surface thermal properties or temperature pattern of different regions of the body in tie-stall or freely moving animals (McCafferty et al. 1998; Weissenboeck et al. 2010; Svejdova et al. 2013). Health, reproductive outcome, and productivity are directly or indirectly linked to body temperature in livestock (St-Pierre et al. 2003; Duff and Galyean 2007) (Figs. 3.2 and 3.3). The skin surface temperature of different body parts varies appreciably (Figs. 3.2 and 3.3). It is suggested that eye temperature may be correlated with core body temperature and exhibited a more uniform temperature than any other anatomical region due to lack of insulation around the eye, and this region is often closest to core temperature compared to other peripheral regions (Teunissen and Daanen 2011). In addition, Pavlidis et al. (2002) demonstrated that posterior area and the caruncula of lacrimalis have rich capillary beds innervated by the sympathetic system and respond to the change in blood flow and stress response. Later, measuring ocular temperature was

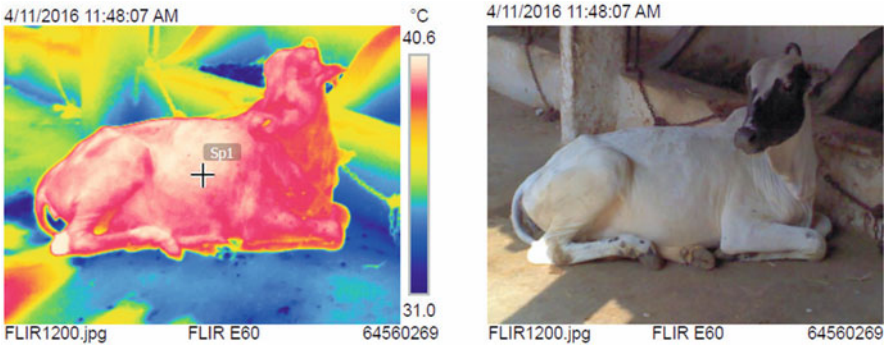

Fig. 3.3 Normal and Infrared thermal image of a same Deoni cow (*Bos indicus*) showing a selected spot (Sp1) temperature of 40.0 °C in a thermogram

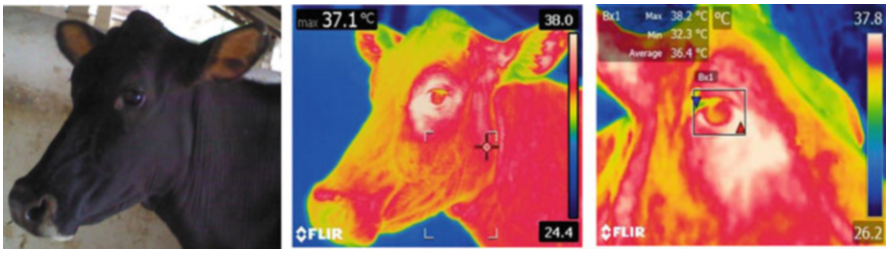

Fig. 3.4 Normal and Infrared thermal image of face of a HF crossbred cow showing a facial skin surface temperature pattern and ocular area temperature and selected spot (caruncula of lacrimalis) showing highest temperature (38.2 °C) in a thermogram

considered for recording core temperature and therefore variability in periorbital region, heat dissipation could be monitored using infrared thermal imaging camera (Dvijesh et al. 2008) (Fig. 3.4).

3.4 An Overview on Application of Infrared Thermography in Livestock and Poultry

IRT was used as a tool to assess hoof health status (Lokesh Babu et al. 2018) of cattle and buffalo, and Cockroft et al. (2000) employed IRT in Friesian heifer for the diagnosis of septic arthritis. Alsaaod and Buscher (2012) observed that the coronary band and the skin above 2 cm from the coronary band temperature were higher in early and mid than the late lactational cows. The tails of the docked heifers remained 1.43 °C warmer than the baseline after cold sensitivity testing, whereas the tails of the intact heifers were only 0.97 °C warmer than the baseline. Schaefer et al. (2004) concluded that IRT to be a highly sensitive indicator of thermal changes in bovine viral diarrhea virus infected animals**.** IRT was employed to measure eye temperature in cattle subjected for cautery disbudding and surgical castration (Stewart et al.

2008) and similarly Willard et al. (2007) easily detected the febrile state using IRT in cattle administered with of bacterial endotoxin (Lipopolysaccharide). Berry et al. (2003) determined the magnitude and pattern of udder temperature variation in HF multiparous cows using IRT. Colak et al. (2008) demonstrated that IRT could be used for early detection of subclinical mastitis in Brown Swiss and Holstein cows. Sathiyabarathi et al. (2016, 2018a, 2018b) demonstrated that IRT could be used effectively for early detection of naturally occurring mastitis (subclinical and clinical form) in HF crossbred, Karan Fries crossbred and Deoni (*Bos indicus*) cows. Infrared thermography was used to detect illness in transition dairy cows, and some IRT variables performed better as a screening test than rectal temperature (Macmillan et al. 2019). Costa et al. (2014) assessed the ability of IRT to detect intramammary infections by measuring USST of lactating dairy ewes. Murat Karaku and Ferda Karaku (2017) used infrared thermography to assess the infection status of metal-tipped, plastic-tipped, and electronic ear tags. Byrne et al. (2019) used infrared thermography to detect hoof infection in sheep. Febrile responses in pigs inoculated with *Actinobacillus pleuropneumoniae* were assessed using IRT (Loughmiller et al. 2001). Similarly, Dewulf et al. (2003) evaluated the possibility of body surface temperature measurements using IRT to predict the pig with fever. Friendship et al. (2009) concluded that scanning groups of penned pigs utilizing a hand-held thermal camera could provide early warning of potential outbreak of *Actinobacillus pleuropneumoniae*. Cook et al. (2015) used infrared thermography to monitor febrile and behavioral responses to vaccination of weaned piglets and suggested that IRT as a disease detection and surveillance tool in swine barns. In mule deer, a considerable rise in temperature as an early sign of foot and mouth disease could be observed as early as two days before using thermograms (Dunbar et al. 2009). Temperature change was monitored using IRT in experimentally induced rabies infection in Raccoons (*Procyon lotor*) (Dunbar and MacCarthy 2006). Jacob et al. (2016) diagnosed pododermatitis in broiler chickens by infrared thermography and observed that there was a decrease in the surface temperature in the center of the lesion when compared to healthy footpad.

3.5 Application of Infrared Thermography in Animal Reproduction

3.5.1 Infrared Thermography in Monitoring Estrous Cycle and Ovulation in Cattle

Accurate detection of estrus and ovulation are the indispensable pre-requisites for successful artificial insemination and optimal reproductive performance. Various methods, viz. teaser, trans-rectal palpation, ultrasonographic examination of ovary and reproductive tract, use of hormonal assays, electrical impedance of vaginal mucus, estrual mucous crystallization pattern, contact devices and remote monitoring devices, etc. for estrus detection. Recently, researchers have focused on temperature profile of vaginal, peri-vulvar, and vulvar temperature using vaginal loggers,

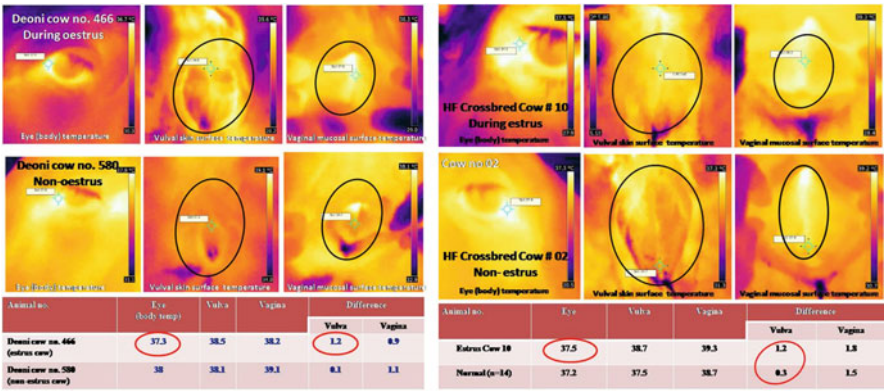

Fig. 3.5 Infrared thermogram showing body temperature (eye temperature), vulval skin surface, and vaginal mucosal surface temperature difference during estrus and non-estrus stage in Deoni (*Bos indicus*) and HF crossbred (*Bos indicus x Bos taurus*) cows (personal observation)

temperature sensors, and infrared thermography as an alternate method for detection of estrus and prediction of ovulation.

The earliest work by Hurnik et al. (1985) on the use of infrared thermography to predict the onset of estrus in HF dairy cows was by observing the changes in body surface temperature. Talukder et al. (2014, 2015) from Australia utilized IRT for detection of estrus and prediction of ovulation in Holstein-Friesian dairy cows and in another study by the same author demonstrated that performing IRT continuously rather than twice in a day improved the estrus detection performance. Hellebrand et al. (2003) suggested that thermography could be used for estrous climax determination in heifers. The vulval surface temperature increased significantly between 3 days before and on the day of ovulation and application of IRT during estrus improved estrus detection rate in cows with silent or normal estrus (Osawa et al. 2004). Similarly, in both *Bos indicus* and *Bos taurus* X *Bos indicus* crossbred, the vulval skin surface temperature increased with a difference of 1.2 °C than eye temperature during estrus (author's personal observation Fig. 3.5).

3.5.2 Infrared Thermography to Monitor Testicular Thermoregulation in Cattle and Buffaloes

The scrotal surface temperature of a bull shows a positive top-to-bottom gradient due to the vascularization pattern with the top warmer than the bottom and is 5–6 °C lower than abdominal temperature (Kastelic et al. 1996a; Kastelic et al. 1997a, 1997b; Brito et al. 2003, 2004; Arteaga et al. 2005). Infrared thermal imaging technique is considered as a most prospective, non-invasive, and non-contact method to study scrotal surface temperature and thermal gradient pattern. Diurnal rhythms did not show any influence on scrotal surface temperature, hence scrotal thermography could be performed at any time of the day (Kastelic et al. 1996a). Sex

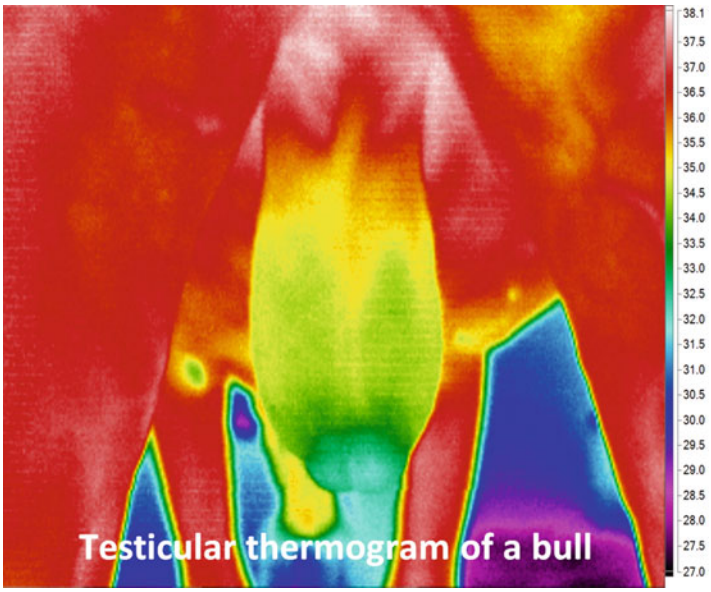

Fig. 3.6 Testicular thermoregulation of a bull

organ diseases accompanied with local temperature changes were diagnosed using infrared thermography (Kozumplik et al. 1989). Testicular thermoregulation of a *Bos indicus* bull shows top-to-bottom thermal gradient vascularization patter with top warmer (white color) to bottom cool (Blue color) (Fig. 3.6).

Kastelic and his co-workers have extensively used infrared thermography to study scrotal and testicular thermoregulation. Scrotal surface temperature from proximal to distal end displayed a significant gradient pattern with a decreasing trend from the neck of the scrotum to the ventral aspect to scrotum. The subcutaneous tissue and testicular parenchyma demonstrated less to negative gradient in comparison to the scrotal surface temperature. However, in comparison to dorsal pole, the ventral pole of the testis was warmer. The caput epididymis was warmer than nearest area of testicular parenchyma while the cauda epididymis was cooler (Kastelic et al. 1995). Studies on influence of environmental factors and animal activity on scrotal surface temperature of bulls by Kastelic et al. (1996a, 1996b) revealed that ambient temperature, feeding and rising, wetness of scrotum may influence IRT reading. In addition, spontaneous and electroejaculation method of semen collection increased scrotal surface temperature. Insulation studies significantly affected the surface, subcutaneous temperature, and increase in intra-testicular temperature yielded defective spermatozoa (Kastelic et al. (1996c, 1997a).

Studies on scrotal thermoregulatory mechanism using IRT revealed the role of scrotum, testes, and testicular artery. Temperature profile of the testicular artery showed more or less a uniform temperature at proximal end than the distal. Interestingly, the testicular artery again cools down before entering the parenchyma

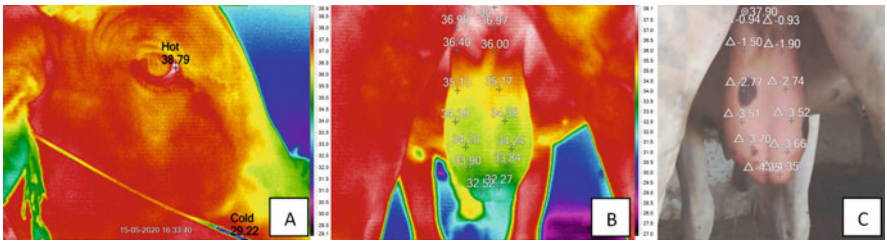

Fig. 3.7 (**a**) Infrared thermogram of a Deoni (*Bos indicus*) breeding bull showing body temperature (eye temperature). (**b**) Scrotal/testicular thermogram showing the surface temperature gradients in from proximal to distal pole of testis. (**c**) Image showing the temperature difference between proximal (proximal temperature as reference value) to distal end of testes and cauda epididymis

(Kastelic et al. 1997b). Effect of GnRH treatment on plasma testosterone concentrations and scrotal surface temperature by Gabor et al. (1998) revealed that GnRH treatment significantly increased plasma testosterone concentrations and usually caused significant increases in scrotal surface temperature measured by IRT.

In breeding bulls reduced fertility could be predictable using scrotal thermogram. Lunstra and Coulter (1997) described that the bulls with sperm abnormality, higher percentage of sperm with proximal droplets, and lower pregnancy rates exhibited abnormal scrotal temperature patterns when compared with bulls with normal or questionable thermograms patterns. Coulter et al. (1997) observed that the bulls on the high-energy diet showed a smaller gradient than those on the moderate-energy diet (3.4 vs 3.9 °C) and the scrotal surface temperature gradient varied significantly without influence on the top, bottom, or average scrotal surface temperature. Recently, Teixeiraa Vanessa et al. (2019) studied the semen quality and scrotal surface temperature of crossbred Girolando (Gyr x Holstein) bulls from two breed compositions using infrared thermography and recommended that scrotal thermogram can aid in the identification of bulls for breeding.

It was interesting to note that breed difference exists on scrotal thermoregulatory mechanism especially Nellore (Bos indicus) bulls showed lower body (ocular area), spermatic cord and proximal area of the scrotum, and scrotal surface temperature than Girolando (Bos taurus taurus x Bos Taurus indicus) bulls. The skin surface temperature of Nellore bulls presented lower temperature and was less influenced by climatic variables compared to Girolando bulls. In *Bos indicus* breeds of cattle IRT of the eye and scrotal surface temperature in Deoni (Fig. 3.7) and Malnad Gidda (Fig. 3.8) (a dwarf breed of cattle) showed a clear temperature difference between eye (body) temperature and scrotal temperature. Interestingly, the scrotal/testicular thermogram showed the temperature gradient pattern from proximal to distal pole of testis including cauda epididymis. Similarly, seminal characteristics also varied between breeds, with the Nellore breed presenting superior semen quality in both fresh and thawed samples than Girolando bulls (Redivo Júnior et al. 2020).

Ahirwar et al. (2017, 2018, 2019) and Satendra Kumar et al. (2019) used infrared thermography to assess scrotal thermoregulation and its association with season,

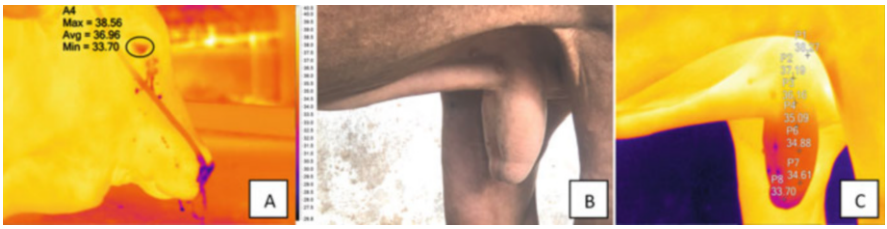

Fig. 3.8 (**a**) Infrared thermogram of Malnad Gidda (*Bos indicus*) breeding bull showing body temperature (eye temperature). (**b**) External genitalia: penis and testes. (**c**) Image showing the temperature difference between proximal to distal end of testes and cauda epididymis

thermal stress, and environment/management factors on semen quality in buffalo bulls.

3.5.3 IRT and Skin Temperature Differences of Peri-Vulvar Area During Estrus in Ewes

Stelletta et al. (2006) explained in his chapter that peri-vulvar temperature of both estrus and anestrus sheep showed a temperature range from 35.9 °C to 37.7 °C with an average of 36.9 °C ± 0.5 °C and from 34.2 °C to 36.5 °C with an average of 35.42 ± 0.63 °C, respectively ($P < 0.05$). The increased peri-vulvar temperature around estrus could be result of an increase in the blood flow to the genital apparatus and adjoining areas associated with endocrine changes.

3.5.4 Application of IRT in Testicular Thermoregulation and Monitoring Estrus in Mares

The increase in peri-vulvar area temperature in mares was positively correlated with estrus and ovulation in mares. Endocrine study associated with changes in peri-vulvar area temperature revealed that increase in serum estrogen levels due to follicular growth was positively correlated with increase in temperature and on the contrary, less serum progesterone around ovulation time was negatively correlated with surface temperature (Stelletta et al. 2012). Bowers et al. (2009) explored the possibility of using thermography to confirm gestation in mares especially around mid to late stage. In stallions, thermography was utilized as a non-invasive tool for assessing the testicular thermoregulatory capability and andrological assessments (Ramires-neto et al. 2012).

3.5.5 Application of IRT for Estrus Detection and Prediction of Ovulation in Pigs

In pigs, vulvar skin temperature and body surface temperature increased 24 to 48 hours before the estrus and was correlated positively with estradiol peak level (Schmidt et al. 2013). Sykes et al. (2012) reported that 1.58 °C increase in vulva temperature in pig and similarly, Simoes et al. (2014) reported 5.3 ± 2.4 °C increase in vulvar temperature during 48 hours before estrus in pig. The relation between vulvar skin temperature and ovulation time in gilts and sows was studied using IRT by Scolari (2010). The results of the study concluded that the sows showed significantly higher vulvar temperatures at the time of estrus period than gilts, but there was no significant difference in vulvar temperatures during ovulation time between the gilts and sows. The most significant increase in temperature was seen at 36 hours before ovulation in gilts and at 24 hours before ovulation in sows. The researcher concluded that the surface temperature of the animal did not vary significantly both during non-estrus and estrus periods, only vulvar temperature was changed.

3.6 Application of IRT in Reproduction of Other Mammals and Birds

Durrant et al. (2006) evaluated the suitability of infrared thermography to detect pregnancy in giant pandas, using the domestic dog as a model for comparative purposes. Pregnancy and pseudopregnancy were successfully detected in giant pandas. Heat stress and thermoregulatory capacity and fertility were correlated in male llamas (Schwalm et al. 2008) using IRT and different physiological measures. Heath et al. (2002) assessed the safety of testicular biopsies in llamas using thermography. Thermography was used to quantify elevated scrotal temperature associated with administration of gonadotropin releasing hormone and elevated testosteronemia in Alpacas (Stelletta et al. 2012). Justyna Cilulko-Dołęga et al. (2018) studied the applicability of thermography during the breeding season and early nursing in farmed fallow deer and concluded that thermal imaging supports estrus detection, but with significant limitations. Thermal imaging does not support early pregnancy detection in fallow deer. Temperature differentials between the examined body parts are reliable indicators of pregnancy only in the last trimester when fetal development is the most rapid.

3.7 Factors Influencing Thermographic Imaging

Thermography should be taken under shaded area, evading straight sunlight and wind movement. Animal's body surface or the site of interest should be free from wetness, dung, and any extraneous materials. There are several environmental factors that influence the body surface temperature of animals such as camera to object distance, wind speed, and solar radiation. The camera to object distance has a

predictable effect on the thermographic temperature. As camera to distance increases the surface temperature decreases. Similarly, wind speed and direct sunlight increase the surface temperature. The IRT temperature is highly dependent on emissivity. So, the wind speed and solar radiation must be taken care of when implementing IRT in disease detection system. Prior to taking IRT image animal must be tied properly in standing position under shaded shed. The area of interest for thermography may be wiped with the clean towel to remove dung and dirt. Mechanical brushing or wiping should be avoided before imaging which may cause changes in body surface temperature. If very essential, resting period for 10–15 min is given prior to imaging after brushing (or) wiping.

3.8 Conclusion

Infrared was discovered in 1800 by Sir William Herschel as a form of radiation beyond red light. These "infrared rays" (infra is the Latin prefix for "below") are used mainly for thermal measurement. Infrared thermography (IRT) is a simple, effective, on-site, and non-invasive method that absorbs surface temperature, which is emitted as infrared radiation and generates a pictorial image without causing radiation exposure. IRT is considered as a potential non-invasive and non-contact diagnostic tool which can be used in veterinary medicine to monitor health, animal welfare, early disease diagnosis, treatment follow-up, zoo animal medicine, etc. and as a remote monitoring tool in precision farming. Recently, modern thermal imaging technology comprises technically advanced thermal cameras with sophisticated software solutions. IRT has potential application in the field of animal reproduction especially monitoring reproductive events, viz. estrous cycle, pregnancy. In males, IRT is considered as a most promising non-invasive and non-contact imaging technique to study scrotal thermoregulation and prediction of reduced fertility associated with testicular pathologies and thermal stress. However, thermography can be routinely applied in animal reproduction as a robust technique only after establishing a species and/or breed specific thermal profile associated with physio-pathological events of interest in reproduction. Establishment of thermographic profile associated with reproductive functions would pave a way for development of temperature associated tool or technology for remote monitoring of reproductive events.

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Technological Advancements for Early Pregnancy Diagnosis in Cattle and Buffaloes

4

Ashok Kumar Mohanty, Sudarshan Kumar, and Manoj Kumar Jena

Abstract

In spite of some remarkable discoveries in the field of bovine pregnancy, the search for a farmer friendly, easy to use pregnancy diagnosis kit in cattle and buffalo is still an unfulfilled dream. The urgency of such a kit can be understood from the fact that whole world is striving hard to develop it since last many decades. In spite of parallel successes in human pregnancy diagnosis way back in early days of year 2000, we have not been able to replicate the same concept in dairy animals. Reason is that challenges were/are many: (1) ruminant placentation is different being synepitheliochorial in contrast to hemochorial in human which may prevent the flow of embryo specific important molecules to maternal circulation in sufficient quantity, (2) the effect of volumetric fluctuation on the detectable concentration of the analyte in urine or blood or milk, and (3) the limited use of highly sophisticated skill and instrumentation for discovery purpose research in animals. Overcoming these challenges, recent past has witnessed quite remarkable achievements in identification, characterization, and validation of important pregnancy-specific molecules using various biological samples for early diagnosis of pregnancy in cows and buffaloes. This chapter delineates the physiology of pregnancy and the potential molecules for pregnancy diagnosis besides the advancements in tools for early pregnancy diagnosis.

A. K. Mohanty (✉)
ICAR-Indian Veterinary Research Institute, Mukteshwar, India

S. Kumar
ICAR-National Dairy Research Institute, Karnal, India

M. K. Jena
Lovely Professional University, Phagwara, India

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Keywords

Early pregnancy diagnosis · Cattle · Buffalo · Pregnancy associated proteins · Kits

4.1 Introduction

The estrous cycle in cow and buffaloes is of 21 days on an average ranging from 18 to 24 days. The cow stands pregnant if the ovum finds a competent sperm within the time span of 24–32 h after ovulation. The difficulty with farmer is that he is unaware of the ovulation as it is a hormonally governed event associated with LH surge in ovary without reflection of very clear and obvious outward signs. The only visible signs of estrus/heat exhibited by the animals are increased mobility, restlessness, bellowing, vaginal discharge, and mounting over companion animals. The pregnancy of the cow depends on the farmer's ability to accurately capture this small window of heat and getting the cow inseminated timely. In artificial insemination process, one insemination in Indian states costs on an average Rs 200–300 (INR). Under normal circumstances, farmers wait for 21 days for "non return to heat." If observable signs of heat are repeated by the animal, the animal is considered nonpregnant. It implies that every such pregnancy failures incur a loss of 21 days of milking from the animal in the cycle of one pregnancy-lactation. From this point of view, ideally a farmer would like to know about the successful establishment of pregnancy immediately after the AI so that in case the animal remains nonpregnant, it can be inseminated again as early as possible without losing much time. At farmer's door, because of limited accessibility to veterinary facilities for induction of heat, a farmer has no choice except to wait for another heat, i.e., after 21 days. This means that if adequate veterinary supports are not available, it is of no use to farmer to know the non-pregnancy status before the non-return day, i.e., 21 days.

But in an organized farm where large number of animals is reared and hormonal synchronization is feasible, the earliest determination of non-pregnancy after the AI can reduce the "wait period" of 21 days (to observe non-return to heat). Currently, due to the lack of a perfect diagnostic system to detect non-pregnancy before 21 days after the AI, the synchronization program needs to wait for at least 25–32 days. Since, the ovary remains in dynamic state of follicular waves, beginning of resynchronization after the first AI irrespective of failure or success of pregnancy from previous AI can be initiated as early as 13 days after the first insemination using synchronization protocol of estradiol benzoate with progestin (synchronization protocol of progestin insert or implant between 13 and 20 days after AI in addition to a second injection of estradiol benzoate (EB) given at insertion and removal of the progestin (Macmillan et al. 1997) treatment. It is beneficial to turn over the dominant follicle without affecting the function of corpus luteum or pregnancy established with a previous artificial insemination (Macmillan et al. 1997; Burke et al. 2000). This means that the resynchronization program cannot be initiated within 13 days of first AI even if the pregnancy diagnostic could tell the non-pregnancy status of

animal. Irrespective of farmer's ability to plan ahead, the need is to know the pregnancy or non-pregnancy as earliest as possible after the AI.

4.2 Physiology of Pregnancy in Bovines

The pregnancy in bovine is usually of 270 days which starts from the day of fertilization till the delivery of a baby calf. It's a very dynamic process which is orchestrated by fine tuning of several endocrine hormones right from the time of fertilization through pregnancy till it ends into parturition. As we studied in the estrus cycle of cows, ovulation results in release of single ova from either of the left or right ovary during the phase of estrus. The presence of a fertilized entity in the ampulo-isthmic junction is sensed by the cells lining the oviduct, and the cow starts responding to the conceptus through sequential and rhythmic changes in its hormonal profile. The first of those few changes ensued immediately after sensing the conceptus is decreased in the level of prostaglandins. With the drop in the prostaglandin, the corpus luteum doesn't receive signal to regress and therefore continues to synthesize and secrete progesterone which stimulates favorable changes in the oviduct and the uterus to support the growth of fetus.

In ruminants, the intimate contact between fetus and uterus occurs as patches where two cellular layers, namely uterine endometrial cells (UEC) and fetal chorionic cells come close to each other and form button like joint anatomical structures called as placentome. The number and placement of these placentomes vary considerably among ruminant species (Hradecký et al. 1988). The fetal cells contributed in placentome are called cotyledons, and the endometrial cells in placentome are called caruncles. Villi of cotyledons interdigitate into the crypts of caruncles forming a highly adherent area through which all cellular and molecular crosstalk takes place. Histomorphology studies reveal that the trophoblast cells in the placentome are of two types, namely (1) uninucleated cells (UTCs) or mononuclear cells and (2) larger binucleated cells (BNCs). The uninucleate cells differentiate continuously into trophoblast giant cells (TGC) throughout the gestation. The binucleate TGCs can be observed at several stages of ploidy because of acytokinetic mitosis (Klisch et al. 1999). The TGCs get fused with the epithelial cells of caruncles which results in formation of trinucleate feto-maternal hybrid cells with short life span (Carvalho et al. 2006). These hybrid cells mediate the release of fetus-derived molecules to the maternal component. It is this fusion of TGC with mononuclear cells of endometrium because of which the placentation in bovine is of synepitheliochorial type (Wathes and Wooding 1980; Wooding 1992; Klisch et al. 1999; Carvalho et al. 2006).

4.2.1 Prostaglandins

The prostaglandin PGF2α is luteolytic in its function which was established since 1969, in rats and guinea pigs. The PGF2α is carried to the ovarian arteries through a local counter-current mechanism between the uterine vein and ovarian artery or from

the uterine lymphatic vessel to nearby ovary (Pharriss and Wyngarden 1969; Blatchley and Donovan 1969; Hixon and Hansel 1974). In the cyclic stages, PGF2α is majorly synthesized and secreted by luteal cells standing high during the early luteal phase which plays its role in the regulation of the estrous cycle. Not surprisingly, the early CL in its 4–5 days after the ovulation remains unresponsive to PGF2alpha (PGF2α). Studies reveal that PGF2α, oxytocin (OT), progesterone (P4), and noradrenalin (NA) have luteotropic function in the CL during its development and maintenance in bovines. These compounds are observed to be strong stimulators of P4 secretion by early- and mid-cycle bovine CL. However, in the late luteal phase, elevated PGF2α level stimulates steroidogenesis and there is production of compounds like endothelin-1, angiopoietin, FGF-2, NO, thrombospondin, TGF-β1, and plasminogen activator inhibitor-B1, which inhibit P4 production, angiogenesis, cell growth, and ECM remodeling in sequential manner to promote regression of CL (Meidan et al. 2017).

Elevated PGF2 alpha concomitant with uterine PGF2 alpha secretion causes luteal regression by increasing cGMP level in luteal cells and thus causing loss of LH receptor. Such events eventually result in both structural and functional regression of CL and fall in the progesterone level. With pregnancy, PGE1 and PGE2 in the endometrium and PGE2 in uterine venous plasma increase. The high ratio of PGE to PGF2alpha during pregnancy makes CL unresponsive to PGF2 alpha and progesterone synthesis and secretion continues to support pregnancy. In cow and also in sheep, the whole gestation is supported by luteal progesterone only with minimal or nearly insignificant role of placental progesterone.

4.2.2 Progesterone

Progesterone is an essential hormone to maintain pregnancy in cows. It is secreted during the luteal phase of estrous cycle with progressive increase in its concentration form after ovulation till luteal PGF2α dominates and makes the CL regressed towards the end of the cycle. During pregnancy, trophoblast plays a crucial role in providing the signals for establishment and continuation of pregnancy, prior to the implantation and placentation (Schuler et al. 2018). It is important to understand that the elongation of conceptus is dependent on the luteal progesterone which regulates gene expression from endometrium necessary for creating a congenial environment for the developing embryo.

4.2.3 Signals from Placenta (Peri-Implantation Trophoblasts)

Prolongation of luteal phase in case of pregnancy in polyestrous spontaneously ovulating species generally depends on some placental origin molecules which are either luteotrophic (human) or antiluteolytic, e.g., domestic ruminants, pig, horse (Spencer and Bazer 2004; Bazer 2015) in nature. In early pregnancy in case of ruminants, the interferon tau (IFNT derived from trophoblast) plays a crucial role in

maintaining the luteal function and said to be initiator of maternal recognition of pregnancy (MRP).

4.2.3.1 IFN-Tau (IFNT)

Uterus receives many antiluteolytic factors from the conceptus. IFNT is a 172-amino acid long polypeptide belonging to type I IFN. It suppresses the expression of oxytocin receptors (OTR), estrogen receptor alpha (ER), and an enzyme cyclooxygenase-2 (COX-2) in endometrial cells. This abrogates the non-pregnancy positive feedback circuit between CL and PGF 2 alpha where PGF2 alpha is supposed to perform luteolytic action on CL to bring it back to follicular phase (Spencer and Hansen 2015; Hansen et al. 2017).

IFN-τ expression is restricted to a small window of time during early pregnancy and with extremely low levels in extrauterine tissues and maternal circulation. IFT although itself being a poor candidate as pregnancy biomarker can however stimulate the expression of a large number of genes essential for supplying nutrition to the endometrium, enhancing receptivity to support implantation, differentiation of endometrial cells, initiation of placentation and localized immunotolerance. A complex network of signaling is also triggered by IFNT in peripheral blood leukocytes that involves IFN-τ stimulated genes (ISG) such as ISG15, MX2, and OAS1 proteins. Steroids are important molecules during normal reproductive stages as well as during pregnancy. Besides the classical estrogens, androgens (produced in lesser quantity), progesterone, some bioactive steroids are also synthesized in placentae which probably exhibit crucial function during pregnancy.

4.2.3.2 Placental Progesterone

Progesterone is very important and indispensable hormone in the maintenance of pregnancy. Many functions associated with pregnancy like differentiation of endometrial tissues, quiescence of myometrial tissues, cervix closure, and immunotolerance in gravid uterus (Chwalisz and Garfield 1997; Spencer and Bazer 2004; Arck et al. 2007). The CL becomes the primary source of P4 in the whole gestation period with rare contribution from placentae in species like goat, pig, and even no placental production of P4 for pregnancy maintenance is there. However, in species like sheep, horse, and human, the placenta takes over the role after species specific time of gestation and luteo-placental shift (Meyer 1994; Mitchell and Taggart 2009). Throughout gestation, the bovine placenta contributes negligible to the maternal progesterone levels which are predominantly of luteal origin. Moreover, no classical nuclear progesterone receptors are found in the fetal part of bovine placentomes (Schuler et al. 1999). However, there are reports which confirm that placental estrogens and P4 are important factors controlling caruncular growth, differentiation, and function (Hoffmann and Schuler 2002).

4.2.3.3 Placental Estrogen

Estrogen can be seen as class of related molecules because different forms of this molecule have been observed at different times of physiological events. Follicular steroidogenesis is generally dominated by estradiol-17β which is the primary

estrogen during pregnancy. Many ungulates have estrone and its sulfonated derivatives (estrone-3-sulfate) dominating the free counterpart concentrations in maternal circulation, as are observed in sheep (Nathanielsz et al. 1982; horse: Hoffmann et al. 1996; cattle: Hoffmann et al. 1997; llama, alpaca: Aba et al. 1998). In human, estriol is the common placental estrogen form. Placenta of cow can produce estrogen independently from cholesterol (Schuler et al. 2008a, b). On the other hand, the placenta of human and horses depend on C19 precursors as there is significantly less expression of CYP17A1gene (Raeside 2017). Regarding role of placental estrogen during pregnancy in primates, there are many reports such as their involvement in differentiation of trophoblasts, regulation of steroidogenesis in placenta, maternal and uteroplacental blood circulation, neovascularization of placenta and developmental process of mammary gland (Pepe and Albrecht 1995). Estrogen receptors (ER) are expressed in dynamic pattern along the pregnancy. Various estrogens may vary in their binding affinities to the nuclear estrogen receptors (ESR) and their paralogues (ESR1 and ESR2). The weak estrogens show agonistic property when strong ESR ligands are absent. However, the weak estrogens can show antagonistic properties when strong ESR ligands are available. The cumulative information tells that although the maternal compartment (uterus, birth canal, mammary gland) are the predominant targets of placental estrogens, their role in placental differentiation cannot be undermined.

In bovines, placental estrogen is involved in birth canal preparation, myometrial excitability, and udder development in last trimester. Presence of CYP19A1 transcripts in the blastocyst of bovines by seventh day of insemination shows the production and functional importance of estrogens from beginning to end of pregnancy. However, the relatively more expression of estrogen receptors on endometrium and specifically on caruncular epithelial cells (Schuler et al. 2002a, b) in placentome indicates that the targets of estrogen are predominantly in mother tissues and organs. A possible role of placental estrogen is seen as stimulator of proliferation in caruncular epithelial cells (Schuler et al. 2000). In cow and other domesticated ruminants, the sulfonated derivatives dominate over the free forms in all stages of pregnancy except the immediate pre- and intrapartal period (Hoffmann et al. 1997). Placental estrogens are sulfonated by estrogen specific sulfotransferase (SULT1E1) in TGC. The interaction with nuclear receptors is abolished due to the sulfonation of estrogens. Additionally, the polarity is increased in the sulfonated forms and that make them different from the lipophilic steroids (free forms), and the sulfonated forms are not able to cross the cell membrane by passive diffusion, as a result of which their distribution is reduced. From diagnosis point of view, the sulfonated derivatives mainly circulate at higher level than their free forms and probably reenter to the free steroid pool by activity of the enzyme steroid sulfatase (STS). The sulfonated derivatives function as substrates for bioactive steroid production locally in particular target tissues (sulfatase pathway of steroidogenesis). The uptake of sulfonated steroids by corresponding transporters and their hydrolysis masks the local control of various activities (Mueller et al. 2015). Conclusively, the CYP19A1 expression in vicinity to each other masks the activity of placental estrogens as local

regulators in placentome of bovines which signifies their involvement in local processes.

4.2.3.4 Placental Androgens

Androgens are the class of steroid molecules that primarily promotes male specific secondary sex characteristics. However, in lose terms they also refer to the metabolites of actual androgens or C19 derived steroids like androstenedione, dehydroepiandrosterone (DHEA), or sulfonated form of DHEA which lack any function at the androgen receptor but may act as precursors for bioactive androgen synthesis. C19 steroids are the common progenitor molecules both for the estrogens and androgens. As there is considerably less CYP17A1 expression, the human and horse placenta rely on extra-placental sources for C19 derived steroids (Raeside 2017), however; placenta of cow (Schuler et al. 2006) and sheep (Mason et al. 1989; Gyomorey et al. 2000) can convert C21 steroids into C19 form for estrogen production. The biochemical changes occurring at the feto-maternal junctions and mutual transport of these androgenic substances therefore can be viewed as an important clue to determine the gender of the fetus by looking at the tilt of steroids in maternal circulation. Obviously, a strict control over transport and amount of bioactive androgens is exercised to avoid sex differentiation in female fetuses and the chances of virilization in mothers. One such mechanism of restriction of biological activity of androgens in human is by aromatization of androgens. Androgen receptors are present in placenta but their exact role is not fully understood. It is speculated that these receptors may exert their function in the independent pathway (not depending on steroids) by constitutive transcription of the N-terminal domains or cross talk with different other signaling pathway (Davey and Grossmann 2016).

4.2.3.5 Chorionic Gonadotrophins

Follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH) are the glycoproteins made up of common alpha subunits and a hormone-specific beta subunit, and they perform an important role in hormonal regulation of gonadal function. LH-related glycoprotein hormones secreted from trophoblast cells (chorionic secretion) are called chorionic gonadotrophins (CG). Human chorionic gonadotrophin (hCG) is made up of the common alpha subunit and a specific β chorionic gonadotropin (βCG) subunit, which is very similar to the human β LH subunit although it is generated from one of the βCG genes. In the beginning of pregnancy, hCG produced from blastocyst is an important signal for the maternal recognition of pregnancy by stimulating and maintaining luteal function through binding of LH receptors till the placenta functions the role of progesterone source. Additionally, hCG is also involved in the angiogenic activities in endometrium, myometrial quiescence, immunotolerance, and controlling the syncytiotrophoblast formation (Perrier d'Hauterive et al. 2007; Fournier et al. 2015). It is important to learn that equine chorionic gonadotrophin (eCG) is completely different from hCG in the sense that beta subunit of eCG is synthesized from βLH gene. Moreover, the eCG is having different and intense glycosylation pattern. On contrary to hCG, the eCG expression starts after the

maternal recognition of pregnancy (day 35–36). Interestingly, BLAST based bioinformatics analyses of hCG with *Bos taurus* nucleotide and protein database do not find any significant match. It implies that hCG-like molecules are absent in bovine. It is noteworthy that highly glycosylated proteins (PAGs) have been identified in the cotyledonary cells at the placentome (Lotfan et al. 2018a, b).

4.2.3.6 Pregnancy-Specific Members of the Prolactin and Growth Hormone from Placenta

Growth hormone (GH) and prolactin (PRL) are structurally related and developed from a common precursor during evolution. The corresponding receptors such as GHR and PRLR are also have similar structures. In ruminants, significant evolution of GH and PRL encoding genes has been occurred. The PRL gene duplication has been observed in ruminants and as a result cluster has been formed having PRL, PL, and PRP genes. Many ruminants including cow have a single GH-like gene with significant evolution even if there is no gene duplication has been observed. In the placentae of bovines, there are no evidence of GH expression.

4.2.3.7 Prolactin

The PRL family proteins of rodents and ruminants with various names are placental lactogen (PL), PRL-like proteins (PLPs), PRL-related proteins (PRPs), and proliferin (PLF). The trophoblast cells express PL in bovines around implantation. The PL and PRP expression in ruminants is generally confined to TGCs after the placentation, and there it is highly upregulated during differentiation of TGC from UTCs and further maintenance after migration of TGCs into epithelia of caruncles or fusion of TGCs with CECs occur (TGC-endometrial heterokaryons). Ideally, the PL of ruminants acts as a regulator in the uterus and mammary gland development and delivery of nutrients from maternal to fetal circulation (Gootwine 2004; Soares 2004; Haig 2008). Prolactin receptors (PRLR) have been found in glandular epithelial cells of the ruminant endometrium which supports endometrial gland differentiation during pregnancy. However, in ovine, PL stimulates uterine milk secretion from endometrial cells into uterus by acting in paracrine manner where it doesn't enter to maternal circulation. In cattle, level of PL is very low (ng/ml) throughout gestation (Wallace 1993) and shows mammogenic effects in steroid primed cows (Byatt et al. 1997). However, PL levels are high in fetal circulation during early days pregnancy (25–30 ng/ml) which declines to 10–20 ng/ml prior to parturition (Hoffmann et al. 1997; Alvarez-Oxiley et al. 2007).

4.2.3.8 Prolactin-Related Protein (PRP)

In addition to PL, in ruminants, another distinct subfamily of PRL-related placental transcripts has been identified which are structurally similar to PL and PRL while the amino acid sequence is quite different from PL and PRL. These are called prolactin-related proteins (PRL). At transcripts levels, more than 10 different isoforms have been documented, while at protein level, only one predominant PRP-1 isoform has been confirmed. Quantitative proteomics-based study of pregnant urine in cows, however, has provided experimental evidences for many other isoforms of PRPs as

well (Bathla et al. 2015; Rawat et al. 2016). The functional status of PRP-1 (produced from TGCs during the whole pregnancy period) is not understood because it does not bind to either PRL or GH receptor. The in vitro findings reveal that it might stimulate angiogenesis in placentomes (Patel et al. 2004a, b, c; Ushizawa et al. 2010).

4.2.3.9 Relaxin/Insulin-like Family Peptides

Relaxin and insulin-like peptides belong to a superfamily of ligands which activate rhodopsin G protein-coupled receptors. In mammals, the RLN/INSL gene family comprised of relaxin 1 (RLN1), relaxin 3 (RLN3), and the genes that encode insulin-like peptides 3–6 (INSL 3–6). The RLN and INSL family members are protein hormones with pleiotropic action in many physiological phenomena. Additionally, RLN1 (human RLNH2), INSL3, INSL4, and INSL6 are involved in reproductive processes in males and females. The RLN3 and INSL5 are primarily involved in the neuroendocrine system and the gut, respectively (Anand-Ivell and Ivell 2014). RLN1 and RNLH2 are generally considered as reproductive relaxins. During pregnancy, CL (in humans, rodents, and pigs) and placenta (in rabbit, dog, cat, camelids, and horse) are responsible for increasing its concentration in maternal circulation throughout gestation. The different physiological functions of RLN are decidualization, modulation of immune system, myometrial quiescence, angiogenic stimulation, mammary gland development, and preparation of birth canal. Presence of RLN receptor RXFP1 in trophoblast cells in human and canine indicates that they a play role locally in placenta.

The RLN1 gene is not present in bovines, whereas a pseudogene is found in goat, sheep, and other ruminants. However, the bovines express functionally active receptors for RLN1 (RXFP1) and INSL3 (RXFP2). Thus, it is assumed that in ruminants, other members of the RLN/INSL family or non-relaxin ligands could compensate for the missing RLN1 (Nowak et al. 2017).

4.3 Pregnancy Diagnosis in Bovine

4.3.1 Ideal Samples

For pregnancy diagnosis, a variety of samples can be used like blood, urine, milk, and saliva. But practically, sample collection from dairy animals is not as simple as in human. Therefore, not surprisingly, urine and saliva although a preferred sample in human doesn't attract same popularity in dairy husbandry.

Milk can be considered as a very ideal sample in dairy animals as it is easily collected by the animal handler and time of sampling can be tightly controlled. But in first time, pregnant animals milk is not available and therefore alternative samples are essential. Milk also suffers from the diurnal volumetric and compositional fluctuation. Blood, urine, and saliva collection impose restraining difficulties and therefore are not preferred choices by the farmers. Urine and saliva are collected non-invasively and therefore preferred over blood. Nevertheless, blood being the

immediate reflector of cellular changes is considered better indicator sample over urine or saliva. Another difficulty with urine sample is that farmers usually have little control over the timed sampling. For example, collecting the first morning urine may prove tedious as the definite time of urination by the animals cannot be ascertained. Moreover, the volumetric fluctuation creates dilution problem in the identification of analyte by any diagnostic technique. Similarly, saliva has its own difficulty of being available in small amount and being contaminated with regurgitated food and secretions of local salivary gland in addition to the circulatory secretions. Therefore, the final decision about the choice of a perfect sample depends on the combined ability of diagnostic assay to identify the target in concentrations as least as possible in the sample as well as on the ease of collecting the true representative sample.

4.3.2 PD by Rectal Palpation

Feeling of bulged uterine horns and the bump of fetus by rectal palpation is an old age method which is still the method of choice of PD in dairy cows and buffaloes. The earliest time when rectal palpation can be done is between 35 and 65 days after the AI. This method doesn't require samples to be collected from the animal and is performed on live animals. It is quick, requires no specialized equipment, gives instant results, and is the most economical of all methods at present. During rectal palpation, usually following parameters are noticed depending on the advancement of pregnancy. During early pregnancy (1–3 months), uterine horn asymmetry, decreased tone of pregnant horn, fluctuating contents (fetal water and vesicle) in pregnant horn, and later on in both horns, palpable CL on ovary in the same side of pregnant horn, fetal membrane slip and amniotic vesicle are appreciable. L**at**e pregnancy (>3 months) is characterized by cervix located anterior to pelvic rim, non-retracting and flaccid uterus, palpable placentomes and fetus (occasionally), and the increased diameter of median uterine artery with detection of fremitus (Table 4.1).

The limitations of this method could be that experienced veterinarians are required to perform rectal palpation. The time of diagnosis is certainly after 35 days of AI. The chances of uterine infection and fetal abortion are high in case due care is not adopted. The diagnosis of positive pregnancy can be misleading in case of presence of pathological contents in the uterus.

4.3.3 Transrectal Ultrasonography

In this technology, a probe is inserted per rectal to examine the uterus. Through ultrasonography, fetus sex determination can also be done. Moreover, through this technique, early pregnancy diagnosis can be done by 28 days of gestation. Therefore, in terms volume of data generated about the reproductive tract and fetus, the ultrasound-based diagnosis remains the first choice with more than 99% accuracy. However, it is costlier. Once the animal restraining and operator -equipment

Table 4.1 Positive signs of pregnancy at rectal palpation

Stage of pregnancy	Membrane slip	Amniotic vesicle	Fetus	Placentomes	Fremitus A. uterine media	
					Ipsilateral	Contralateral
30 days	±	+				
45 days	+	+				
60 days	+	+				
75 days	+	+		+		
90 days	+		+	+		
105 days			+	+	+	
4 months			+	+	+	
5 months			+	+	+	+
6 months				+	+	+
7 months				+	+ +	+

proficiency is optimized, the speed of diagnosis may be even better than that of rectal palpation.

Sector and linear-array, real-time [B-mode (brightness modality)], ultrasound devices are commonly available for veterinary applications. In general, linear-array transducers are used with linear-array imagers, and sector transducers are used with sector scanners. The equipment for ultrasound scanning includes a transducer and a linear-array scanner. Transducers of different frequency are used. It is observed that a transducer with 3.0 MHz frequency gives more tissue penetration but minimum details, whereas a transducer with 7.5 MHz frequency gives minimum tissue penetration but maximum resolution. In general, a transducer of 5.0 MHz frequency provides reasonably detailed images of ovary and uterus (Rajamahendran et al. 1994). Badtram et al. (1991) conducted ultrasonography (5 MHz probe) in 200 Holstein cows and heifers at day 16–day 31 following A.I. The accuracy of the study was found to be 62.5%, 55.6% sensitivity, 70.2% specificity, and a prediction value of 67.8%. Moreover, the accuracy and sensitivity were observed to be less in pregnancy diagnosis of earlier stage (day 16 to day 22) (50% and 25%, respectively) as compared to that at a later stage (day 23 to day 31) (70.2% and 68.8%, respectively) after A.I (Badtram et al. 1991).

4.3.4 Blood Test

4.3.4.1 Pregnancy-Specific Protein (PSP)

Pregnancy-specific proteins are family of five members of glycoproteins secreted by trophoblast cells, and PSPB is an acidic glycoprotein with several isoelectric variants around pIs of 4.0–4.4, and apparent molecular weight of this protein is 78 kDa (Sasser et al. 1989a, b). It is found elevated in cattle serum at approximately 28 days of gestation and lasts until calving. One disadvantage to PSP based diagnosis is that PSP level remains high after calving for an extended period (60 days). It, therefore, requires caution while interpreting the test result by either following a waiting period

after calving or to use the test only in those situations where the AI has been done only after 60 days of parturition. It cannot determine the age or gender of the fetus. The use of this protein remained restricted to the research lab where radioimmunoassay (RIA) based methods were developed to detect pregnancy in cows around 30 days of pregnancy (Humblot 1988 review article).

4.3.4.2 Early Pregnancy Factor (EPF)

EPF has been detected in the serum of pregnant cows within 48 h of pregnancy (Cordoba et al. 2001). IT is believed to initiate immuno-tolerance in the mother. The EPF is associated with rosette inhibition test (RIT) in blood of cow (Nathanielsz et al. 1982) which is too cumbersome to perform and analyze; therefore, the diagnostic potential is poor. A study indicated that RIT ($P < 0.05$) has the potential to distinguish pregnant from nonpregnant dairy cows in the first week of pregnancy (Laleh et al. 2008). Cordoba et al. (2001) concluded that ECF test is an unreliable method for determining pregnancy status of dairy cattle on day 6 after estrus. However, the interesting part of the test using EPF was that the test was a colorimetric and qualitative assay on lateral flow platform that used monoclonal and polyclonal antibodies incorporated onto a nitrocellulose membrane from Concepto Diagnostics, Knoxville, TN. An antibody-gold conjugate was used to detect the presence of the ECF glycoprotein. An RIT-based study in bovine finds that the measurement of EPF activity is useful for monitoring the viability of bovine embryos (Sakonju et al. 1993).

4.3.4.3 Pregnancy Associated Glycoproteins (PAGs)

PAGs constitute a family of more than 300 related isoforms. All of them belong to the aspartic proteinase family like pepsin, cathepsin D and E. The expression pattern of individual isoforms from the conceptus varies with time. Therefore, it is not very clear which isoform may serve as a better indicator of pregnancy. In a very comprehensive review on "Placental PAGs: Gene origins, expression patterns and use as markers of pregnancy" written by (Wallace et al. 2015), it has been clarified that earlier pregnancy-specific molecules like PSP60, PSPB, (synonymous to PAG1) EPF, etc. are the members of PAG family. Early research by Patel et al. (2004b) focused on PAG1 and PAG9. They demonstrated that bovine *PAG9* message was approximately ten times greater in abundance early in gestation (days 30 and 60) compared with the message for bovine *PAG1*. Touzard et al. (2013) however demonstrated that the pattern of expression of these isoforms is more dynamic than thought when observed holistically. They reported that modern PAGs are two to four times more abundant in cotyledons compared with intercotyledonary chorion. In contrast, most of the ancient PAG transcripts they tested were elevated in the intercotyledonary chorion compared with cotyledons.

In bovine, PAG specific transcript was detected by day 15, and two transcripts were identified on day 18. One, boPAG2, from the PAG II group and other was previously unknown, boPAG22, closest to boPAG2 (Garbayo et al. 2008). This study, however, is limited in its ability to clearly say about the truly most abundant isoform as it used gene specific primers from the available sequences at the database

which might have caused primer sequence related biasness in amplification of a particular isoform.

In a similar approach to discover specific PAG isoform during early pregnancy in buffaloes, Lotfan et al. (2018a) performed cDNA cloning of large number of PCR amplicons produced from RNA isolated from the cotyledons of 30 d, 45 d, and 90 day pregnancy and screened more than 300 colonies randomly. They used equimolar mixture of different primers to cover 22 isoforms of PAGs. PAG-7 was found to be the most abundant transcript/isoform in early pregnancy at around 30 days of pregnancy.

A study carried out in 220 Holstein Friesian cows using RIA-based method to detect PAG in blood and milk samples concludes that PAG concentration in milk and blood increases after 28 day of pregnancy with rapid increase near the parturition. However, the PAG level falls down faster in the milk than in the blood. The limit of detection by RIA using double antibody was 0.2 ng/ml (Zoli et al. 1992) and they quantified PAG in maternal peripheral blood (using both plasma and serum) at day 22 of pregnancy (mean +/− SD, 0.38 +/− 0.13 ng/ml) in some animals and at day 30 in all pregnant cows. Reese et al. (2018) have reported that serum PAG concentrations can be quantified using ELISA at 24th day of gestation. Filho et al. (2020), measured PAG concentration in beef cattle blood by ELISA and found that serum concentration of PAG at day 24 ($\geq$0.33 ng/mL in cows) and ($\geq$0.54 ng/mL in heifers) was 95% accurate at determining pregnancy status at day 30 of gestation. Mean circulating concentration of PAG at day 24 in pregnant versus nonpregnant cows was found to be (1.69 $\pm$ 0.10 ng/mL vs 0.30 ng/mL $\pm$ 0.07 ng/mL; mean $\pm$ SEM, respectively, at $P < 0.001$.

There are many variations of immunodiagnostics for detecting PAG. In a modified version, Friedrich and Holtz (2010) used competitive double antibody ELISA using a polyclonal anti-bPAG-IgG and an anti-rabbit-IgG raised in sheep for coating. They concluded that pregnancy can reliably be diagnosed from day 28 onwards in serum and from day 150 onwards in milk. In a study (Karen et al. 2015) that compared different forms of immunodiagnostic platforms to detect PAG in milk and blood, PAG ELISA was found to be a suitable alternative to TRUS and PAG-RIA giving similar results. The test results that data of these three methods are shown in Tables 4.2 and 4.3.

Table 4.2 Efficiency of different method of pregnancy diagnosis in bovines

	Sensitivity to diagnose pregnant cows at day 28	Specificity to diagnose nonpregnant at day 28	Overall accuracy
TRUS	92.7%,	91.5%	92%
pAG-RIA	100%	94.4%	97%
PAG ELISA	90.2%	98.3%	95%

Study carried on blind samples of 41 confirmed pregnant and 59 confirmed nonpregnant cows with $n = 100$.

Table 4.3 Bovine pregnancy rapid test (UbioquickVET; BPRT) versus ELISA-PAG test (Bovine Pregnancy Test DG29®) (Moussafir et al. 2018)

	Sensitivity to diagnose pregnant cows between 30 and 40 days	Specificity to diagnose nonpregnant between 30 and 40 days	Overall accuracy
BPRT	89.4%	89.8%	90%
DG29	100%	81.3%	94%

Study carried on blind samples with n = 212 cows with TRUS as gold standard final confirmatory of pregnancy.

In a similar study by Dufour et al. (2017), IDEXX milk pregnancy test (IDEXX laboratories Inc., Westbrook, ME, USA) which is based on the principle of PAG ELISA is supposed to give equal accuracy to that of TRUS when tests are done during 28–45 days of pregnancy. Ricci et al. showed that the concentration of PAGs in blood and milk of pregnant cows gradually rose from day 25 on and reached its first peak on day 32 after fertilization was reduced again until day 60, and then began to rise constantly until day 273. The concentration of PAGs in the blood of pregnant cows reached 4.48 ± 0.92 ng/mL on day 28 after fertilization, compared with 0.2 ng/mL in nonpregnant cows (Wang et al. 2020). A study by Green et al. (2000) validated an ELISA that specifically targeted PAGs secreted early in gestation that had a shorter half-life (4.3 days vs. 8.4 days) than the previous targets to reduce the potential for false positives in postpartum cows (Table 4.4). The ELISA was demonstrated to accurately detect pregnant cows via serum concentrations of PAGs at day 28 post-insemination.

4.3.4.4 PAG as Indicator of Embryonic Mortality

Since, PAG originates from placental trophoblast cells; its concentration in the maternal circulation is directly related to the presence or absence of embryo. The PAG concentration falls rapidly with embryonic loss. Pohler et al. (2016) found in their study that in lactating dairy cows, circulating concentrations of PAG on d 31 of gestation may serve as a good marker for predicting EM between d 31 and 59 of gestation.

4.4 Early Pregnancy Associated Transcripts from Uterus and Embryo

The embryo of cattle enters the uterus from ampulo-isthmic junction (the site of fertilization) at about the 16-cell stage which coincides with day 4 of pregnancy. It subsequently forms a morula, at which stage the first cell-to-cell tight junctions are formed. By day 7, the embryo progresses to blastocyst consisting of an inner cell mass (ICM), which after further differentiation gives rise to the fetus, and the trophectoderm, which ultimately contributes to the placenta. Early pregnancy window therefore can be considered primarily both from physiological and diagnosis standpoint, to be of 30 days from the time of fertilization.

Table 4.4 Comparison of the performance of three methods: rectal palpation, TRUS, and PAG

Method	Accuracy	Specificity (%)	Sensitivity (%)	Positive predictive value	Negative predictive value (%)	Authors
Rectal examination Single pregnancy						
31–35	37.5	90.9				Karen et al. (2015)
45–50	93.8					
51–55	100	100	96			
In total	37.5–100	90.9–100				
Twin pregnancy	49.4	99.4		86.1		Day et al. (1995)
USG						
In total	82	73	78.2	80.8	74.4	Racewicz et al. (2016)
21 (heifers)		87.5	50			Romano et al. (2006)
26 (heifers)		94	100			
24 (cows)		96.6	74.5			
27 (cows)		96.6	100			
PAG						
In total		95.5	99.2	99.8	80.8	LeBlanc (2013)
57.6		94.6	94.3			Garmo et al. (2008)
19,024		92.0	11.1			Karen et al. (2007)
31			100			
19–55		90.0–100	100			

A large number of studies have generated data on the mRNA profile both from uterus and embryo during the onset of pregnancy. This information has improved our understanding about the feto-maternal cross talk for pregnancy recognition, implantation signaling, growth, development, and nutritional support. Such knowledge simultaneously has enabled deeper investigation into these molecules (transcripts/proteins/metabolites) in order to find close and specific association with pregnancy and identification thereof in the maternal circulation from viewpoint of biomarker discovery for pregnancy diagnosis. The early pregnancy period, right from the fertilization can be divided into two time segments: i) preimplantation period starting from 0 day, i.e., from the time of fertilization to 7–8 days and the second ii) the peri-implantation period starting from eighth day onward till 16–18 days of pregnancy which signifies the time of hatching of the blastocyst from the zona pellucida and its subsequent elongation coincident with the time of maternal recognition of pregnancy (MRP). Microarray and RNAseq technologies have enabled the identification and relative quantitation of a large number of transcripts (mRNA, non-coding RNA, etc.) simultaneously from a small amount of tissue or fluid.

Readers are encouraged to read an excellent review on genes, proteins, and pathways involved in the endometrium or uterine lumen that potentially regulates peri-implantation blastocyst growth in cattle by Spencer et al. (2008). And a similar review can be found useful on gene expression in elongating and gastrulating ruminant embryos by Hue et al. (2007). In a comparative study of different developmental stages of embryo at days 7, 14, 21, 28, reported expression of 680 genes which were upregulated and 26 genes downregulated from day 7 to day 14 which included enzymes, transcriptional regulators, oncogenes, tumor suppression, cell cycle control, and apoptosis. The major temporal changes occur in the embryo transcriptome between the blastocyst stage on day 7 and the initiation of conceptus elongation on day 13 (Mamo et al. 2012). The differential transcriptome is extensively regulated by the environment to which the embryo is exposed up to the blastocyst stage (i.e., in vivo compared to in vitro). The list of 465 differentially expressed mRNA transcripts between seventh day embryo and 13thdays embryo is shown in the table in which 180 genes are upregulated and 285 genes are downregulated. These transcripts are supposed to play their role during embryo elongation. RNAseq experiment revealed trophoblast Kunitz domain proteins, pregnancy associated glycoproteins, cytoskeletal transcripts, heat shock proteins, calcium-binding proteins, APOA1, AHSG, BOP1, TMSB10, CALR, APOE, TPT1, BSG, FETUB, MYL6, GNB2L1, PRDX1, PRF1, IFNT, and FTH1 as the most prevalent 20 genes that appear during seventh day till 19th day of embryo development (Table 4.5).

4.4.1 Interferon-Stimulated Genes

Interferon-stimulated genes increase in circulating leukocytes between days 16 and 20 post-insemination (Gifford et al. 2007a, b). Microarray analysis further indicated

Table 4.5 Twenty most prevalent upregulated genes at each developmental stage based on expression intensity as measured by RPKM[a] values (Mamo et al. 2011)

Day 7	Day 10	Day 13	Day 16	Day 19
HSP70	*TMSB10*	*TMSB10*	*TKDP4*	*TKDP4*
TMSB10	*TKDP4*	*KRT18*	*TMSB10*	*TMSB10*
KRT18	*HSP70*	*HSP70*	*APOA1*	*APOA1*
TKDP4	*KRT18*	*FTH1*	*KRT19*	*KRT19*
KRT8	*KRT8*	*KRT19*	*TKDP3*	*KRT8*
EIF5A	*APOA1*	*HSPB1*	*KRT8*	*TKDP3*
PAG11	*KRT19*	*KRT8*	*HSPB1*	*AHSG*
DNAJB1	*HSPB1*	*APOA1*	*AHSG*	*BOP1*
CLIC1	*TPT1*	*TPT1*	*FETUB*	*HSPB1*
S100A14	*MYL6*	*HMGA1*	*KRT18*	*CALR*
TPT1	*PAG11*	*S100A14*	*APOE*	*APOE*
CFL1	*GNB2L1*	*EIF5A*	*TKDP2*	*TPT1*
CALR	*S100A14*	*CALR*	*CALR*	*BSG*
GNB2L1	*PRF1*	*EF2*	*RBP4*	*FETUB*
EF2	*EF2*	*PKLR*	*BSG*	*MYL6*
MYL6	*BSG*	*SLCA3*	*PRF1*	*GNB2L1*
BSG	*CLIC1*	*PRDX1*	*FTH1*	*PRDX1*
APOA1	*CALR*	*MYL6*	*GNB2L1*	*PRF1*
PRF1	*EIF5A*	*TKDP4*	*PAG2*	*IFNT2*
HMGA1	*PRDX1*	*PRF1*	*SPARC*	*FTH1*

that many genes, including IFN-τ stimulated, are upregulated during early pregnancy. Green et al. (year) have however shown that the differential expression of such genes is influenced by the parity of the animal, being more definite in heifers as compared to multiparous animals. All these experiments have suggested IFN-τ stimulated genes to be potential pregnancy detection biomarkers; still there is no field level test available based on these markers (Balhara et al. 2013).

4.4.2 miRNAs as Potential Biomarkers for Pregnancy Diagnosis

MicroRNAs (miRNA) are now being targeted as simple and available biomarkers for various diseases and physiological states. The miRNAs are 18-22 nucleotides long and regulate the expression of various genes and their detectable range has been found in biological fluids, e.g., serum, amniotic fluid, urine, milk, etc. Study on mare fetal sample revealed 7 miRs expressed in pregnant animals as compared to that of nonpregnant ones (Ncube et al. 2011). Additionally, in the pregnant and cyclic sheep, miR is observed in the uterine fluid. Additionally, study of Ioannidis and Donadeu (2016) showed 6 miRs (day 16: bta-miR-26a, bta-miR-29c, bta-miR-138, bta-miR-204. Day 24: bta-miR-1249, day 16 & 24: hsa-miR-4532) having differential expression in pregnant heifers. Preliminary data indicate that there is significant

correlation between the level of miRNA at days 17 and 24 with gestation and the level falls down with the loss of embryo (Reese et al. 2018).

4.5 Advanced Computer Aided Data Collection and Use in Pregnancy Diagnosis

The advancement in our ability to collect and handle large amount of data simultaneously assisted with high-throughput information technology has made it possible to record gross physiological changes including movement of the animals, rectal temperature, respiration, water and feed intake, etc. over a big population. The significant correlations have been reported with these data and specific physiological state of the animal. The pedometer, based on an acceleration sensor, has been used to detect the activity of cows, and it enables automatic diagnosis of estrus and pregnancy. Gil et al. showed that in 90% of pregnant cows, the temperature of milk and rectum was 0.64 and 0.46 °C higher, respectively. However, in nonpregnant cows during 5–12 days after insemination, there was no obvious change between nonpregnant and empty cows. Zhang et al. found that 1 month before parturition, the rectal temperature of pregnant Holstein cows was higher than that of nonpregnant cows, gradually increasing on average to 39.18 °C 7–8 days before parturition, and subsequently reached a peak temperature of 39.32 °C on the third day before parturition. The temperature variation is correlated with reproduction state, such as estrus, pregnancy, and parturition (Wang et al. 2020).

4.6 Proteomics-Based Studies to Discover Early Pregnancy Biomarkers

Mass spectrometry has greatly helped in our ability to discern the total proteome profile of any sample. The low abundant proteins which are missed by the conventional diagnostic techniques are captured with much success using MS. Bathla et al. (2015) investigated urine of Karan Fries cows and reported more than 1550 proteins. Later, Rawat et al. (2016) in an attempt to identify protein-based biomarker during 16–25 days of pregnancy in Karan Fries cows conducted a DIGE experiment on urine which revealed a total of 11 differentially expressed proteins out of which nine were upregulated having fold change $\geq$1.5. The Label Free Quantitation (LFQ) revealed 195 differentially expressed proteins between pregnant and nonpregnant urine samples.

Label free quantitative proteomics-based discovery in milk whey from pregnant and nonpregnant cows at 21 days of pregnancy revealed apolipoprotein B (1.48 fold), spermadhesin 1 (1.36 fold), and perilipin (1.19 fold) as upregulated proteins in comparison to 21 day cycling cows. In the same study using extracellular vesicle (EV) enriched milk whey, authors identified ten proteins (polymeric immunoglobulin receptor, 6-phosphogluconate dehydrogenase, decarboxylating, sulfhydryl oxidase, mucin-1, signal recognition particle receptor subunit alpha, lymphocyte

antigen 96, glyceraldehyde-3-phosphate dehydrogenase, folate receptor alpha, G protein-coupled receptor class C group 5 member B and hedgehog interacting protein-like 2) as differentially expressed (Johnston et al. 2018). In these studies, however, the fold change recorded was very minimal being in the range of 1.18–1.34.

In a DiGE based study, Lee et al. (2015) on serum of two pregnant cows at 21 post-AI revealed 13 protein as differentially expressed in the pregnant serum, among which 7 proteins were upregulated proteins such as conglutinin precursor, modified bovine fibrinogen and IgG1, and 6 proteins were downregulated proteins such as hemoglobin, complement component 3, bovine fibrinogen, and IgG2a. Jin et al. in (2005) performed proteomics analysis of serum samples of Holstein dairy cattle at 21 and 35 days after AI of pregnant and nonpregnant and reported profiles of proteins involved in early pregnancy and suggested the potentiality of these proteins to detect early pregnancy in bovine. Out of all the potential, pregnancy-specific proteins identified were IgG2a heavy chain constant region, transferrin, immunoglobulin gamma heavy chain variable region, and albumin. Proteomics of milk samples from pregnant and nonpregnant cows revealed 16 protein spots, out of which 14 pregnancy-specific spots were upregulated and 2 spots downregulated in the pregnant milk sample (Han et al. 2008).

Proteomics analysis of serum of pregnant and nonpregnant buffaloes in early pregnancy elicited that synaptojanin-1, apolipoprotein A-1, Keratin 10, apolipoprotein, and Band Von Willebrand factors were differentially expressed in between the groups (Balhara et al. 2013). Forde et al. (2014) studied to characterize the global changes in the composition of the uterine luminal fluid (ULF) from pregnant heifers during pregnancy recognition (day 16) using nano-LC MS/MS. On 16th day, 1652 peptides were identified in the ULF by nano-LC MS/MS. Of the most abundant proteins present, iTRAQ analysis revealed that RPB4, TIMP2, and GC had the same expression pattern as IFNT, while the abundance of IDH1, CST6, and GDI2 decreased on either day 16 or 19. ALDOA, CO3, GSN, HSP90A1, SERPINA31, and VCN proteins decreased on day 13 compared with day 10 but subsequently increased on day 16 ($P < 0.05$). Purine nucleoside phosphorylase (PNP) and HSPA8 decreased on day 13, increased on day 16, and decreased and increased on day 19 ($P < 0.05$). The abundance of CATD, CO3, CST6, GDA, GELS, IDHC, PNPH, and TIMP2 mRNAs was greater ($P < 0.001$) in the endometrium than in the conceptus. By contrast, the abundance of ACTB, ALDOA, ALDR, CAP1, CATB, CATG, GD1B, HSP7C, HSP90A, RET4, and TERA was greater ($P < 0.05$) in the conceptus than in the endometrium.

Romero et al. (2017) performed proteome and mass spectrometry analysis in ovine uteri to identify metabolites and proteins in uterine flushing (UF) that may contribute to nourishing the conceptus. Proteome analysis detected 783 proteins present by days 14–16 of pregnancy in UF, and the prominent proteins are annexin (ANX) A1, A2, and A5; calcium-binding protein (S100A11); profilin 1; trophoblast Kunitz domain protein 1 (TKDP); and interferon tau (IFNT). These proteins function in endocytosis, exocytosis, calcium signaling, and inhibition of prostaglandins (annexins and S100A11); protecting against maternal proteases (TKDP); remodeling

cytoskeleton (profilin 1); and altering uterine release of prostaglandin F2 alpha as well as inducing IFNT-stimulated genes in the endometrium and the corpus luteum (IFNT). Readers are encouraged to consult a comprehensive review on technical approaches used in proteomics-based biomarker discovery for early diagnosis of pregnancy in bovine by Buragohain (2017).

4.7 Commercially Available Kits

4.7.1 Progesterone Based

Several kits are available based on the concentration of progesterone in milk (Wang et al. 2020). Cow-side milk progesterone assays conducted between 18 and 24 days post-AI had a reported accuracy of 97.2% for cows identified as nonpregnant (Pennington et al. 1985). Radioimmunoassay (RIA) is a good method to detect progesterone concentration in milk. Although RIA shows a higher detection accuracy of pregnancy and non-pregnancy during the early stages of pregnancy, the expensive equipment and the radioactive hazards make it difficult to apply extensively.

4.7.2 Enzyme Immunoassays (EIA) Using P4

EIA is based on the principle of ELISA where enzymes are used in place of isotopes in RIA. The progesterone antibody is conjugated with enzyme horse radish peroxidase (HRP) which in turn reacts with suitable substrate tetramethylbenzidine (TMB) to produce colored reaction product. The intensity of color produced is directly proportional to the amount of progesterone antigen present in the tested sample. Bayemi et al. (2007) used EIA-based study to detect progesterone in milk. The EIA kit achieved an accuracy of the pregnancy diagnosis of 85.4% and an accuracy of the non-pregnancy diagnosis of 98.3% (Wang et al. 2020). Some of the commercially available kits include Accufirm RPT, B.E.S.T, Calfcheck, Enzygnost, EstruCHEK, Open Alert BoviPro21, OvuSure Rapid Tube, and Target.

4.7.3 Kit Based on Estrone Sulfate

An EIA in microtiter plates has been developed for rapid solid-phase measurement of estrone sulfate in bovine milk without extraction. The assay was 99% and 86% accurate in predicting pregnant and nonpregnant animals, respectively. The estrone sulfate-based assays, however, are not popular because of wide fluctuation in its level being influenced by genetic makeup, weight, parity status, and environment (Sasser and Ruder 1987; Bekele et al. 2016).

Table 4.6 Early conception factor (ECF) test predicted pregnancy rate, agreement with actual pregnancy rate and the proportions of false positive, true negative, and false negative results at varying time intervals post-breeding

Sample/ day	ECF-predicted pregnancy rate	ECF agreement with actual pregnancy rate	True negative[a]	False positive[a]	False negative[a]
Serum[b]					
3	87.5	52.5	60.0	89.5	10.5
9	95.0	50.0	100.0	100.0	0.0
15	85.0	45.0	0.0	90.9	9.1
21	90.0	50.0	50.0	90.0	10.0
30	100.0	50.0	0.0	100.0	0.0
Milk[b]					
3	70.0	45.0	41.7	68.2	31.8
9	82.5	52.5	57.1	84.2	15.8
15	57.5	57.5	58.8	58.8	41.2
21	75.0	50.0	50.0	75.0	25.0
30	10.0	37.5	44.0	16.7	83.3

[a] True negative: a negative ECF test for a confirmed nonpregnant cow relative to the total number of nonpregnant animals; False positive: a positive ECF test acquired for a confirmed nonpregnant cow relative to the total number of disagreements between ECF testing and the actual pregnancy rate; and False negative: a negative ECF test acquired for a confirmed pregnant cow relative to the total number of disagreements between ECF testing and actual pregnancy rate

[b] Comparisons between serum and milk ECF-predicted pregnancy rates differed on respective day, $P < 0.05$.

4.7.4 Kits Based on EPF

To replace RIT, a more user-friendly assay was developed for rapid detection of EPF in the serum and milk of cattle within 1–15 days after breeding. This test, referred to as the early conception factor test, works on the principle of lateral flow assays that use monoclonal and polyclonal antibodies incorporated into nitrocellulose membranes in which an antibody-gold conjugate is used to mark the presence of the EPF glycoprotein (Threlfall and Bilderback 1998). This diagnostic technology is analogous to other currently available lateral flow qualitative tests for hCG, LH, and other hormonal indicators or disease reactants (Gandy et al. 2001). Extensive study on the effectiveness of the commercial ECF test for diagnosing non-pregnancy revealed a high degree of non-reliability of the test, wherein only 44.4% and 55.6% of the confirmed non- pregnant heifers were identified correctly by serum ECF analysis at days 1 to 3 and days 7 to 9 after AI, respectively (Table 4.6; Balhara et al. 2013).

4.7.5 PAG-Based Tests

Commercial PAG tests are currently available using both milk and blood samples. At the recommended sampling time, all commercial tests provide 98–99% true positive

(pregnant) reading and false positive (pregnant but open) rates ranging from 1 to 5% however, some variation may be due to late embryonic mortality (Reese et al. 2018).

4.8 Conclusion

Early pregnancy diagnosis in cattle and buffalo has been a challenging task always. Although a number of test kits are been available, none of them is user friendly and has their inherent disadvantages unlike in case of human being. There is no LFA kit for testing at the farmers door yet. Recent advancement in proteomics and metabolomics technique has facilitated faster discovery of potential biomarkers for early pregnancy diagnosis which may in the short to medium term will prove highly suitable for development of field usable early pregnancy diagnostic kit.

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Bull Sperm Selection for Assisted Reproduction

5

Jane M. Morrell and A. Kumaresan

Abstract

The sperm population within an ejaculate is heterogeneous, reflecting differing ability to fertilize an oocyte. Selecting sperm sub-populations with certain desired characteristics may have a positive effect on pregnancy rate in assisted reproduction. This review describes different methods of biomimetic selection for bull spermatozoa, focussing particularly on colloid centrifugation. Migration, microfluidics and magnetic activated cell sorting are also described. Several versions of the colloid centrifugation technique known as single layer centrifugation are available, differing in the volume of sperm sample to be processed. Samples can be processed in volumes ranging from 0.25 to 150 mL, in appropriately sized tubes. Processing small volumes of semen (0.25 mL–1.0 mL semen on 1 mL colloid) is best done in a 15 mL tube, since the interface between the semen and colloid is greater than in a 1.5 mL tube. In addition to current applications, future uses of colloid centrifugation are described, for example, as biomarkers of fertility, for improving the semen quality of young bulls, and for removal of pathogens.

Keywords

Biomimetic selection · Colloid centrifugation · Fertilizing ability · Reproductive efficiency · Livestock production

J. M. Morrell (✉)
Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden
e-mail: jane.morrell@slu.se

A. Kumaresan
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

A. Kumaresan, A. K. Srivastava (eds.), *Frontier Technologies in Bovine Reproduction*, https://doi.org/10.1007/978-981-19-3072-0_5

5.1 Introduction

Selection of bull spermatozoa for assisted reproduction has been reported frequently over the last three decades. The reasons for wanting to select spermatozoa vary from selection of X- or Y-chromosome bearing spermatozoa and thus to choose the sex of the calf before conception, to selecting the spermatozoa that are judged most likely to be able to reach the oviducts and fertilize an oocyte. Extensive reviews have focussed on sperm sex selection, which will therefore not be covered in the present review. Instead, this review will focus on biomimetic selection of the spermatozoa that are most capable of reaching the oocyte. The highlights and changes that have occurred over the last 30 years will be described, and the directions of potential future development of various methods will be discussed. The first section will consider the assisted reproduction technologies (ART) for which bovine spermatozoa are used and why sperm selection is needed for them, followed by a description of the different sperm selection methods available. The remainder of the review will focus on developments in one of these methods: colloid centrifugation.

5.2 Assisted Reproduction in Cattle

Artificial insemination (AI) is by far the most widely used reproductive biotechnology for breeding cattle. Estimates of the number of AIs performed in cattle are difficult to pinpoint because of differences in the way such figures are reported but in 1999, 264 million bull semen doses were produced worldwide (Thibier and Wagner 2002). It is likely that this figure has more than doubled in the last 20 years since the annual production of cattle and buffalo semen doses in India alone is now 115 million. Embryo transfer is the second most widely used ART, but accounts for a much smaller proportion of the animals bred than AI. In 2019, almost 1.5 million transferrable embryos were produced (Viana 2019). The embryos used are either derived in vivo or may be produced in vitro in some countries.

Another reproductive biotechnology, intracytoplasmic sperm injection (ICSI) of bovine oocytes, is possible (Magata et al. 2019) but is not performed routinely. There are problems with the technique in cattle, partly because of the dark ooplasm, making visualization of the internal structures of the oocyte difficult (Wei and Fukui 2020), and the large head of bull spermatozoa (Galli et al. 2003), but also due to lack of oocyte activation. These technical difficulties result in low efficiency of the technique in cattle (Unnikrishnan et al. 2021).

Embryo production in vitro (IVP) and ICSI have their own specific requirements for sperm preparation.

5.2.1 Sperm Preparation for Artificial Insemination

Bull semen receives minimal preparation; if the ejaculate reaches certain thresholds, e.g. for sperm membrane integrity, or morphology or motility, a suitable extender is

added to give the desired sperm concentration, calculated to deliver a set number of spermatozoa in a 250 μL straw. Different breeding companies have their own thresholds of acceptance and their own values for the optimum sperm number per dose. The extender usually contains a cryoprotectant such as glycerol if the semen is to be frozen, and antibiotics.

5.2.2 Sperm Preparation for Embryo Transfer

In ET, in vivo derived embryos may be produced by performing AI in a superovulated female, followed a week or so later by flushing to recover the resulting embryos. Alternatively, the embryos are produced in vitro (IVP), either following in vitro maturation (IVM) of immature oocytes aspirated from ovaries obtained from the slaughterhouse, or using mature oocytes obtained from a donor cow using ovum pick-up. Whatever the source of the oocytes, some form of sperm selection is performed, both to separate the spermatozoa from seminal plasma (which contains de-capacitation factors) and from the cryomedium in which they were frozen, as well as to select the most robust spermatozoa. Cryopreservation can inflict considerable injury on sperm samples; therefore, sperm selection separates the least damaged spermatozoa from the rest of the sample.

5.2.3 Sperm Preparation for Intracytoplasmic Sperm Injection

The sperm sample for ICSI is first prepared in the same way as a sample for IVF, but then an individual spermatozoon is selected. The operator chooses spermatozoa that are motile and with no apparent morphological defect. The tail is broken, and the spermatozoa are aspirated into the injection pipette before being inserted into the cytoplasm of the oocyte.

5.3 Why Select Spermatozoa?

Ejaculated semen contains a heterogeneous population of spermatozoa at different stages of maturity. These spermatozoa differ in their ability to reach an oocyte after deposition in the female reproductive tract. Selection mechanisms exist within the female to ensure that only the spermatozoa with certain attributes reach the oocyte for potential fertilization (Suarez 2007). Spermatozoa must be motile (to swim against fluid flow), have normal morphology, intact membranes and an intact acrosome. They must also be able to capacitate and to undergo the acrosome reaction in the presence of an oocyte. Thus, of the millions of spermatozoa present in the ejaculate, only a few hundred actually reach the oocyte and only one is able to fertilize it. In assisted reproduction, these natural selection mechanisms may be circumvented, for example, by depositing the semen in a different part of the reproductive tract than would occur during natural mating, resulting in a higher

proportion of the sperm population potentially being able to achieve fertilization. If the oocyte is fertilized by a spermatozoon that is abnormal, development may be impaired or interrupted at some stage, even after implantation. Clearly, in IVP and especially in ICSI, these physiological interactions with the reproductive tract of the cow are missing. Therefore, there is a chance that spermatozoa with sub-optimal characteristics might fertilize the oocyte.

Another reason for utilizing sperm selection is to choose spermatozoa with intact chromatin. Sperm DNA is in a condensed form (chromatin) that becomes increasingly tightly packed as the spermatozoon matures. A spermatozoon with damaged chromatin may have good motility and normal morphology and can compete with other spermatozoa to fertilize the oocyte. The oocyte has the ability to repair some DNA damage but the repair mechanisms may be overwhelmed if too much damage is present (González-Marín et al. 2012). Fertilization by a spermatozoon with damaged DNA can lead to subsequent embryonic development being halted. Some research suggests that, at least in pigs, spermatozoa with damaged chromatin do not reach the oviducts (Ardon et al. 2008), but studies in human patients (Flamigni and Coticchio 2006) suggest that this is not the case since an association was found between DNA fragmentation and post-implantation embryo loss. In horses, an negative association was observed between DNA fragmentation and fertility (Love and Kenney 1998).

The main reason for using sperm selection in ART is to ensure that most of the "unsuitable" spermatozoa are removed, thus enabling high quality spermatozoa to be available to fertilize the oocyte. Since the sperm selection techniques used in the laboratory mimic those occurring in the female reproductive tract, they are known as biomimetic.

5.4 Biomimetic Sperm Selection Methods

Sperm selection methods are based on separation of spermatozoa with certain physical characteristics; they have been reviewed in detail previously (Morrell and Rodriguez-Martinez 2009, 2010, 2016). Recently new methods, such as Magnetic Activated Cell Sorting (MACS) and microfluidic devices have become available which deserve consideration. Therefore, the focus of this section will be on these newer techniques, as well as migration and colloid centrifugation.

5.4.1 Sperm Selection by Migration

The sperm selection methods that have been consistently used in practice are sperm migration (in the form of "swim-up") and colloid centrifugation. These techniques are used when preparing bovine spermatozoa for IVF and enable the spermatozoa to be separated from seminal plasma and extender. In "swim-up", selection is based only on motility; there is no selection for normal morphology or intact chromatin. In contrast, colloid centrifugation selects for morphologically normal, motile

spermatozoa with intact chromatin (Morrell et al. 2009), and also removes seminal plasma proteins that are coating the surface of the spermatozoa (Kruse et al. 2011). "Swim-up" takes approximately 45–60 min, recovering 10–20% of the spermatozoa in the sample. Colloid centrifugation, in contrast, requires only 25 min preparation time (including the 20-min centrifugation) with a recovery rate of >50% for sperm samples transported overnight from the bull stud (Goodla et al. 2014), depending on the sperm quality of the original sample. Although migration methods were the first selection methods to be used when preparing spermatozoa for IVF, only a small proportion of the spermatozoa are recovered, and the procedure takes too long to be of use when preparing whole ejaculates for AI.

Another migration technique is potentially able to select spermatozoa exhibiting a particular type of motility, the ability to move against fluid flow or rheotaxis. Spermatozoa must move against the direction of fluid flow in the female reproductive tract (which flows caudally) to enable them to reach the site of fertilization. Recently, the characteristics of sperm sub-populations separated by rheotaxis were examined (Rappa et al. 2018). Although the velocity of spermatozoa exhibiting rheotaxis flow was different to those that did not, sperm morphology and hyaluronic acid binding did not differ between the two sub-populations. This microfluidic device may be useful as a research tool for investigating which characteristics are relevant for spermatozoa to move within the female tract, but it would not be practical in its present form for routine selection of spermatozoa with high velocity for AI. An AI trial involving low doses of microfluidic-sorted sperm samples ($<10^6$ spermatozoa) was reported to give a pregnancy rate of 37% compared to almost 40% with a standard dose of unsorted controls (Nagata et al. 2018). Chromatin integrity was better, and high mitochondrial membrane potential was higher in the selected samples than in controls. However, a higher proportion of the spermatozoa were starting to capacitate in the microfluidic-selected samples than controls. These results are intriguing, raising the possibility that if the method could be scaled–up and speeded up to process more spermatozoa in a reasonable time, and capacitation could be avoided, it could be feasible to prepare sperm samples for AI in this manner.

5.4.2 Sperm Selection by Magnetic Activated Cell Sorting

Magnetic activated cell sorting (MACS) selects cells on the basis of binding of surface molecules to coated magnetic beads. Thus, spermatozoa that have phosphatidylserine exteriorized on their membranes because they are entering apoptosis will bind to Annexin V- coated magnetic particles and can then be removed from the sample in a magnetic field (Faezah et al. 2014). Although technically feasible for sperm samples, MACS does not appear to have been widely adopted. There are some reports of its use to prepare human spermatozoa for intracytoplasmic sperm injection (ICSI), although no improvements in fertilization, pregnancy, embryo quality, implantation, and live birth rates were observed between controls and selected sperm samples (Nadalini et al. 2014; Romany et al. 2010). Other studies used magnetic nanoparticles coated either with antibody to ubiquitin or with lectin

that binds to glycan exposed on the sperm surface. Similar conception rates were obtained for the nanoparticle-purified sperm samples and the controls, even though the sperm number was half the normal dose in the selected samples (Odhiambo et al. 2014). Similarly, cat or bull spermatozoa selected using nanoparticles were reported to have similar fertilization rates to controls in IVF (Durfey et al. 2019). The technique was considered to require further refinement (Nagata et al. 2018). These results are to be expected since the spermatozoa that are being removed are dead or damaged and would not succeed in binding to the oocyte in IVF. A more relevant aspect to investigate would be whether sperm quality deteriorated more slowly in the selected sperm samples than in controls, because of the removal of damaged spermatozoa that are potential sources of reactive oxygen species.

5.4.3 Sperm Selection by Colloid Centrifugation

This technique has received considerable interest from the equine semen industry, where problems with sperm quality can be encountered since stallions are selected as breeding sires based on their performance in competition rather than on seminal attributes. However, interest in colloid centrifugation from the bovine semen industry has been slight, mainly because bull sperm quality is generally considered to be good. In most countries, there is no market for bull sperm samples that do not survive freezing (possibly with the exception of New Zealand and Ireland where fresh semen is inseminated), resulting in continual selection for ejaculates that reach certain thresholds of post-thaw sperm quality. However, it is possible that the criteria commonly used for accepting bull semen for commercial distribution are not the most relevant in terms of fertility, since only approximately 40% of inseminated dairy cows produce a calf. Various combinations of sperm quality parameters have been suggested as biomarkers of fertility (Kumaresan et al. 2017) that may serve as a better indicator of in vivo fertility than the ones currently employed.

The principle of colloid centrifugation of spermatozoa is that mature motile spermatozoa with intact membranes and good chromatin integrity are separated from the rest of the ejaculate by passage through a colloid during gentle centrifugation (Morrell and Rodriguez-Martinez 2009). Poorly motile or immotile spermatozoa and those with damaged membranes are retained at the interface between the colloid and the semen, provided that the centrifugal force used is low and the centrifugation time is restricted (typically 300 g and 20 min). A detailed description of the protocol for stallion semen was provided previously (Morrell and Nunes 2018). The method for bull semen is similar except that the semen should first be extended to a sperm concentration of approximately 50×10^6 spermatozoa/mL (Nongbua et al. 2017). The selection is based on the ease with which the spermatozoa can pass between the colloid particles; motile spermatozoa will pass through the colloid more easily than immotile spermatozoa, as will spermatozoa with normal morphology and intact acrosomes. Selection also occurs according to density. In the nucleus, the chromatin becomes more and more tightly packed as spermatozoa mature, resulting in an increase in density. Thus, those spermatozoa

Table 5.1 Comparison of migration methods, magnetic activated cell sorting, microfluidics and colloid centrifugation for improving bovine sperm quality

Method	Advantage	Disadvantage
Swim-up	Cheap; readily available; improves fertility	Lose most of the spermatozoa; time consuming, need an incubator and possibly a centrifuge
Microfluidics	Good selection	Low yield; need specialist devices; takes time; only suitable for IVF or possibly low-dose AI; expensive? Effect on fertility unknown. Needs further development
Magnetic activated cell sorting	Rapid	Columns of magnetic particles are expensive; cannot process large volumes of ejaculate; need a magnet, i.e. not readily available in sperm labs. Does not improve fertility
Colloid centrifugation	Good selection; available for all; improves fertility	Colloid is expensive; need a centrifuge

that are less dense remain at the semen/colloid interface whilst the spermatozoa with mature chromatin pass through the colloid. Spermatozoa in which DNA strand breaks are present may be less dense than those with intact DNA although this speculation has not been investigated. Therefore, the sperm pellet will be enriched for motile spermatozoa with normal morphology and intact chromatin, compared to the original sperm sample.

A summary of the different methods of sperm selection discussed in this review is shown in Table 5.1.

Some of the uses of colloid centrifugation in processing bull sperm will now be considered.

5.5 Uses of Colloid Centrifugation in Bovine Assisted Reproduction Technologies

5.5.1 Early Uses of Colloid Centrifugation

Some of the first reported uses of colloid centrifugation in bovine ART were in preparing sperm samples for IVF using Percoll density gradients (de Vries and Colenbrander 1990). The outcome was mixed, with some reports mentioning an increase in the number of acrosome-reacted spermatozoa in the density gradient prepared samples. The problem may have arisen from the use of synthetic human tubal fluid (HTF) to prepare the different colloids needed for the density gradient, since the osmolarity of the resulting colloids may have been conducive for capacitation and acrosome reaction to occur. Using colloid formulations with optimized physical characteristics should avoid this issue (Morrell and Wallgren 2011). A further problem was that a toxic effect of some batches of Percoll was reported, necessitating testing of each batch before use (Avery and Greve 1995). Since then there has been a move to using silane-coated silica instead of polyvinylpyrrolidone

(PVP)-coated silica as the basis of the colloid formulations; the PVP-coated silica preparations at the time could not be autoclaved, which may have been a contributing factor to a detrimental effect perceived with some batches. No further reports of toxicity of Percoll to bull spermatozoa appeared although problems with murine zygote development were observed following exposure of the sperm to PVP (Mizuno et al. 2002).

The next development in colloid centrifugation was the modification of the density gradient technique to use only one layer of colloid, i.e. only one density of colloid and therefore not a density gradient. This modification was described for animal semen in 2006; its use has increased dramatically since then. The advantages of using only one layer of colloid is that there is no need to prepare several colloids of different densities (Morrell and Rodriguez-Martinez 2009) and the ready-to-use species-specific colloid formulations available allow consistency between batches (Morrell 2006). Crucially for its use with animal semen, the SLC technique can be scaled-up to process larger volumes of semen. Although the bull ejaculate is typically only a few millilitres in volume, the sperm concentration is very high, requiring considerable extension before colloid centrifugation. It is important not to overload the colloid if high recovery rates are to be achieved, to minimize sperm competition to enter the colloid (Morrell et al. 2010). Theoretically, a whole bull ejaculate could be processed in approximately eight 50-mL centrifuge tubes, depending on the sperm concentration in the original ejaculate.

Currently, the major use of colloid centrifugation for bovine sperm samples is still in sperm preparation for IVF. Freshly ejaculated bull semen is usually of very good quality, in terms of motility, membrane integrity and morphology. Therefore, semen producing centres do not perceive the need to select spermatozoa by colloid centrifugation, since this method inevitably results in the loss of some robust spermatozoa along with the poorly motile ones. However, an association between chromatin integrity and fertility was reported for Norwegian Red bulls (Narud et al. 2021), showing similar trends to the associations seen in the stallion (Love and Kenney 1998). If an oocyte is fertilized by a spermatozoon with damaged chromatin, embryonic development may be initiated but is halted at some stage (Gopalkrishnan et al. 2000; Lazaros et al. 2011), possibly even after implantation has occurred. In studies with human IVF and ET, implantation rates were lower where the sperm donor had higher rates of damaged chromatin than where the sperm chromatin was normal (Simon et al. 2014). Therefore, we can speculate that selecting for bull spermatozoa with intact chromatin for AI might be beneficial in avoiding some early embryonic loss, which is high in cattle. In a recent study in Swedish dairy cattle, approximately 45% of pregnancies were lost in the early stages (Ask-Gullstrand et al. 2020); the chromatin integrity status of the sperm samples was not reported. It would be interesting to establish whether there is an association between chromatin status and early embryonic loss.

5.5.2 Colloid Centrifugation to Improve Sperm Quality Pre-Freezing

Despite the high quality of most bull ejaculates, it is still possible to improve some aspects of sperm quality further by colloid centrifugation. Goodla et al. (2014) reported less DNA fragmentation and a higher mitochondrial membrane potential in bull sperm samples processed by SLC than controls. Normal morphology was increased in four of the 20 bulls in her study (Goodla et al. 2014). Membrane integrity and total and progressive motility were not different between SLC and control samples. Production of some reactive oxygen species was also increased: thus, there was a higher proportion of spermatozoa producing superoxide, corresponding to the higher mitochondrial membrane potential observed in the SLC samples, but also a higher production of hydrogen peroxide, although levels were very small in all samples. In summary, although there was a less obvious beneficial effect of SLC in bull semen than reported for stallion semen (Morrell et al. 2010), there was still an improvement in some parameters of sperm quality. This result may be due to the quality of bull semen being high to start with.

5.5.3 Colloid Centrifugation to Improve Sperm Quality Post-Freezing

Similar beneficial effects of preparing bull ejaculates by SLC prior to freezing on post-thaw sperm quality were reported (Nongbua et al. 2017). Thus, SLC samples had better chromatin integrity and a greater proportion of spermatozoa with high mitochondrial activity post thaw than controls. However, a study on bulls in Thailand showed that both sperm motility and normal morphology were greater in SLC samples than in controls. These results suggest that the degree of improvement seen depends on the initial quality of the samples, with a greater beneficial effect in the lower quality samples. This result is in keeping with studies on stallion semen (Morrell et al. 2010). In an AI study carried out in Thailand, the pregnancy rate following insemination with SLC-selected samples was approximately twice that of control samples (Thanapol Nongbua, personal communication).

Chromatin integrity can be evaluated in many ways. Apart from detecting single strand breaks, as in the Sperm Chromatin Structure Assay, evaluating free thiols indicates the degree of condensation of the chromatin, while the presence of retained histones has been associated with lower fertility and also indicates potential sites for epigenetic change. Preliminary results indicated that there were fewer free thiols in SLC samples than in controls (Morrell et al. 2017).

5.5.4 Colloid Centrifugation to Prepare Thawed Spermatozoa for IVF

The first stage in preparing spermatozoa for IVF is to separate the spermatozoa from medium and seminal plasma. Colloid centrifugation has several advantages over swim-up for this purpose since it allows selection of the most robust spermatozoa, which are assumed to be the most capable of fertilization. Whereas swim-up (or other migration techniques) enables motile spermatozoa to be separated from the rest of the sample, the spermatozoa may not have other desirable attributes such as normal morphology or intact chromatin (Samardzija et al. 2006). However, IVF cannot mimic fertilization in vivo, not the least because the spermatozoa do not need to traverse the whole of the reproductive tract in order to locate an oocyte. The spermatozoa compete with each other to fertilize the oocytes in the same drop of medium. Furthermore, protocols for bovine IVF are designed to produce the maximum number of fertilized oocytes, including using an excess of spermatozoa. Using either swim-up or colloid centrifugation selects motile or good quality spermatozoa, respectively, thus negating the effect of the quality of the original sample (Morrell et al. 2016). Thus, if the purpose of the IVF is to look for potential differences in fertilizing ability between different sperm treatments, it is important to reduce the number of spermatozoa used to a threshold level so that differences in fertilizing ability can be detected (Sabés-Alsina et al. 2020).

Another interesting development is the use of smaller and smaller centrifuge tubes in an effort to reduce costs. Thus, there are several reports of "mini-gradients" in the literature. A study comparing sperm yield from different sizes of colloid preparations, however, deduced that the recovery rate is greater if 1 mL colloid is used in a 15 mL tube instead of a 1.5 mL (Eppendorf-type) tube (Abraham et al. 2016). This could be an important finding if sperm numbers are limited, e.g. when using commercial sexed semen for IVF. Another point to note is that many protocols using mini-colloid preparations use very high g forces to try to maximize sperm yield. Studies in other species showed that high g forces are associated with considerable DNA damage. Since spermatozoa with damaged DNA can compete with "normal" spermatozoa to fertilize an oocyte, and embryo development can proceed for some time before being halted because of problems with male DNA, it would seem to be preferable to avoid causing DNA damage during sperm preparation. However, no studies have been done to date to show an association with early embryonic death in vivo after transfer of these embryos.

The different version of SLC are depicted in Fig. 5.1 and their uses are summarized in Table 5.2.

Table 5.2 Summary of different versions of single layer centrifugation processing whole ejaculates or sperm samples

Sample	Size of tube (mL)	Volume of sample (mL)	Volume of colloid (mL)	Potential purpose
Thawed straw	15	0.25–1.0	1.0	IVF, ICSI
Aliquot	15	1.0–4.5	4	IVF, AI
Aliquot	50	20	15	AI
Whole ejaculate	500	Up to 200	150	AI

Note: *AI* artificial insemination, *IVF* in vitro fertilization, *ICSI* intracytoplasmic sperm injection.

5.6 Future Uses of Colloid Centrifugation

5.6.1 Colloid Centrifugation as an Indicator of Fertility

The number of spermatozoa passing through the colloid reflects the sperm quality of the original ejaculate. A study with stallion spermatozoa showed that the recovery rate of motile spermatozoa is strongly associated with sperm quality and also with the fertility of the uncentrifuged ejaculate in artificial insemination (Morrell et al. 2014). In a small study with bull semen samples, in which extended semen was sent overnight to the laboratory at 6 °C and SLC was performed on arrival, the recovery rate (proportion of the loading dose appearing in the sperm pellet) was found to vary among ejaculates (mean ± SD 49 ± 18%). This figure is quite similar to the general non-return rates of 40–45% currently reported for dairy cattle in Sweden (Ask-Gullstrand et al. 2020), based on 12,219 inseminations. This preliminary result indicates that it might be possible to use the recovery rate after colloid centrifugation as an indicator of the potential fertility of bull ejaculates. This is an interesting possibility and requires following up.

5.6.2 Improving the Sperm Quality of Young Bulls

Genomic selection enables interesting candidates for future breeding sires to be identified at a young age (Meuwissen et al. 2001). There is considerable interest from breeding companies in being able to use these young bulls as semen donors for AI as soon as possible. However, the sperm concentration, and motility (Murphy et al. 2018) and also sperm morphology (Karabinus et al. 1990) of young bulls tend to be inferior to older bulls. Preliminary results with colloid centrifugation suggest that it could enable some ejaculates to be used earlier than currently possible.

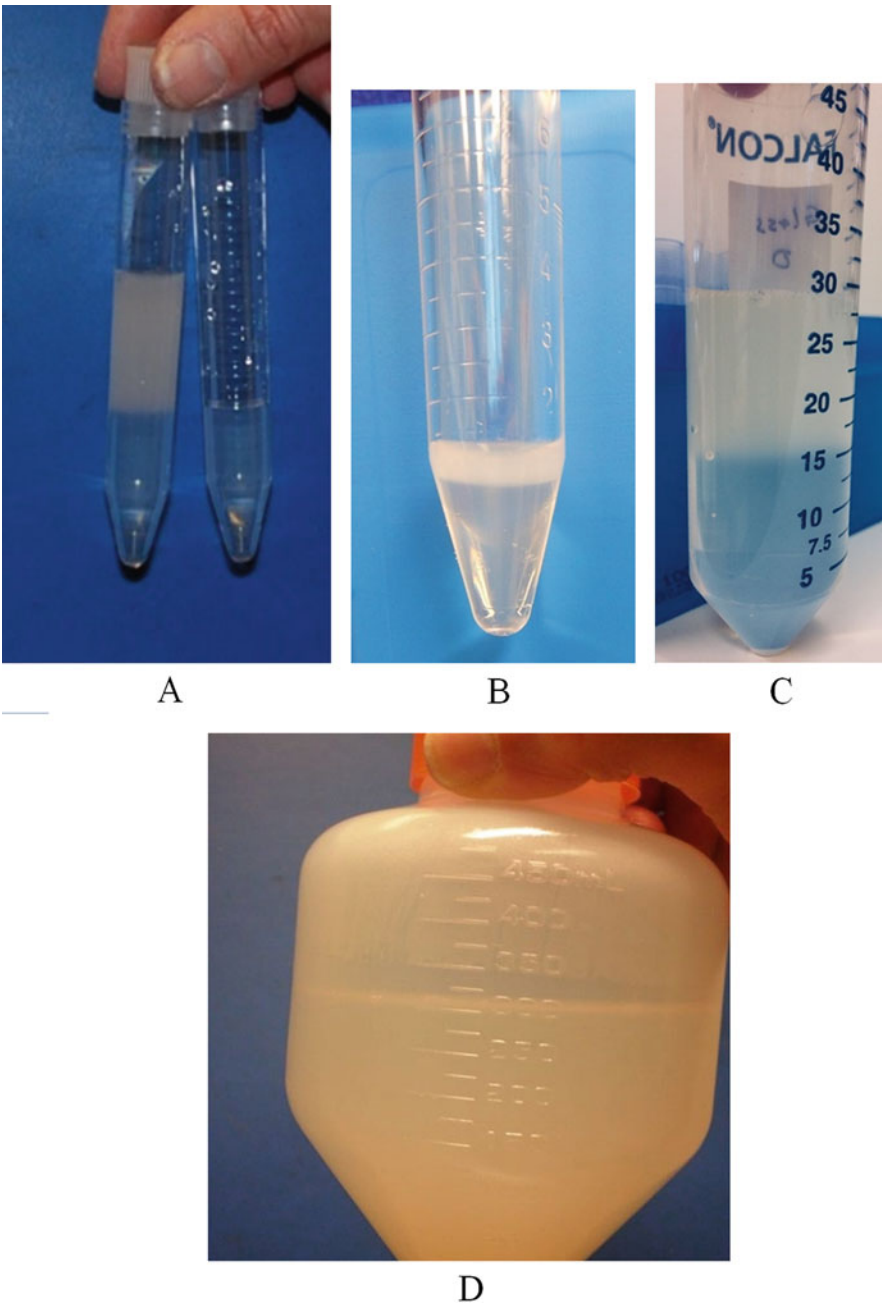

Fig. 5.1 Single Layer Centrifugation (SLC) in tubes of different sizes: (**a**) Small SLC, before and after centrifugation in 12 mL tubes; in the left-hand tube, 4.5 mL extended semen is layered over 4 mL colloid. In the right-hand tube, the sperm pellet is clearly visible. The white line at the interface between the colloid and the seminal plasma and extender contains spermatozoa that have not been able to pass into the colloid. (**b**) Mini-SLC, consisting of 1 mL colloid and 0.25 mL thawed semen before centrifugation. (**c**) Large SLC comprising 15 mL Bovicoll and up to 20 mL extended

5.6.3 Removal of Seminal Plasma

Although much effort has been devoted to determining links between sperm quality and fertility, the contribution of the fluid portion of semen, i.e. seminal plasma, has often been neglected. Recent studies showed that seminal plasma can affect the viability of bovine epithelial endometrial cells in culture and the release of pro-inflammatory cytokines in a fertility-dependent manner (Nongbua et al. 2018a, b, 2020). Seminal plasma from bulls of lower fertility had a detrimental effect on the cultured cells, whereas seminal plasma from bulls of high fertility had either no effect or a less deleterious effect. During natural mating in cattle, semen is deposited in the vagina (Alghamdi et al. 2009), and the spermatozoa move away from seminal plasma through the cervix. Thus, the seminal plasma itself does not enter the uterus. During artificial insemination, however, the semen dose is deposited at the entrance of the uterus, or just inside the cervical canal, and therefore uterine cells are exposed to seminal plasma (Bromfield 2016; Robertson 2005). We speculate, therefore, that the seminal plasma from lower fertility bulls may disrupt the normal signaling mechanisms that are thought to occur at mating to prepare the uterus to receive the embryo several days later. If this is the case, it should be possible to improve fertility of semen doses from bulls of low fertility by removing the seminal plasma using colloid centrifugation prior to freezing. If the non-return rates of the bulls are unknown, it might prove to be better to remove the seminal plasma from all samples anyway. The possibility of using the scaled-up versions of SLC, i.e. in 50 mL centrifuge tubes or larger, would expedite such preparation at the semen collection facility.

5.6.4 Separation of Spermatozoa from Pathogens in Semen

The development of AI occurred in an effort to control disease transmission among herds, in which it was highly successful. However, it is still possible to transmit viruses and bacteria in semen. Bulls for breeding are tested before entering the semen collection program and at regular intervals thereafter. In addition, frozen semen is usually quarantined for 28 days before use, to ensure that the donor was healthy at the time of semen collection. Despite rigorous serological testing of bulls, it is still possible for a male to be seronegative for a particular virus while shedding the virus in his semen, as occurs for bovine diarrhoea virus (Givens 2018). Alternatively, after a recent infection, the animal may shed high virus loads before mounting a serological response and can infect females via this route.

Studies based on colloid centrifugation followed by a "swim-up" procedure removed more than 99% of porcine circovirus particles from spiked semen samples

Fig. 5.1 (continued) semen before centrifugation. (**d**) Extra-large SLC, comprising 150 colloid and up to 200 mL extended semen in a 500-mL tube

(Blomqvist et al. 2011) and the majority of virus particles from semen of stallions with equine arteritis virus infection (Morrell et al. 2013). It is not known whether the remaining virus load would be sufficient to cause infection in inseminated animals since the threshold for infectivity for each virus is not known. Moreover, the establishment of infection may depend on intrinsic factors in the female, such as immune status, number of previous pregnancies, underlying health status. Therefore, further research is needed to see if the remaining virus can be inactivated to ensure that the selected sperm samples are not infective.

Apart from viruses, bacteria can also be present in semen. Bacteria from the skin and the animal's environment colonize the mucosa of the distal reproductive tract and contaminate the semen as it is ejaculated (Rota et al. 2011). The semen extender added to nourish and protect the spermatozoa during freezing acts as a nutrient medium for bacteria and could result in high numbers of bacteria being transferred to the uterus during artificial insemination. Therefore, antibiotics are added during semen processing, as stipulated in national and international legislation (Council of Europe, Directive 92/65/EEC. 1992) but this non-therapeutic application may be contrary to recommendations on the prudent use of antibiotics to avoid the development of antimicrobial resistance. Studies are currently underway to determine whether passing the spermatozoa through a low density colloid to separate all the spermatozoa from the seminal plasma would also remove the bacteria. This method produced promising results with boar semen (Morrell et al. 2019).

5.7 Conclusions

There are several biomimetic methods for bull sperm selection that are appropriate to prepare small volumes of semen but, at present, only colloid centrifugation in the form of single layer centrifugation is relevant for preparing whole bull ejaculates. This method is already used in the equine semen industry and could be used to advantage in the bull semen industry but its widespread use in processing bull semen has not been reported. In contrast, colloid centrifugation is frequently used to prepare bull sperm samples for IVF. Other methods of sperm selection, such as microfluidics, are promising but require further development and/or scaling-up if they are to be both economic and practical for widespread use.

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Nano Purification of Semen: A Novel Technique for Enrichment of Superior Quality Spermatozoa

6

Nilendu Paul, Thirumala Rao Talluri, Pradeep Nag, Kathan Raval, and A. Kumaresan

Abstract

Semen quality is important for achieving high fertility in the bovines. However, semen quality is variable because all the spermatozoa in an ejaculate are not similar. Bull ejaculates contain heterogeneous population of spermatozoa with varying degrees of structural and functional differentiation and phenotypic characteristics. Although several studies demonstrated the existence of subpopulations in an ejaculate, and few subpopulations are superior in terms of quality and fertilizing potential, enrichment or removal of a particular subpopulation to improve the fertility has not been attempted until recently. In the recent past, several techniques have been developed for separation of inferior quality spermatozoa so that good quality spermatozoa can be used for breeding. Among these techniques, the technique of cell separation using nanoparticles based magnetic activation gained momentum. The principle behind this is to coat the iron nanoparticles with suitable antibodies or lectins for targeted capture and removal of desired or undesired cell populations. Several studies have shown that high-quality spermatozoa can be enriched using this technique. This chapter gives an idea about the commonly employed sperm selection techniques and provides a detailed description of sperm purification using nanoparticles. The preparation of nanoparticles, conjugation with molecules of interest and its application in sperm selection (positive and negative) are also discussed.

N. Paul · T. R. Talluri · P. Nag · K. Raval · A. Kumaresan (✉)
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India
e-mail: A.Kumaresan@icar.gov.in

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Keywords

Semen quality · Nano technology · Sperm subpopulations · Enrichment · Bull fertility

6.1 Introduction

Artificial insemination (AI) is considered as the most successful assisted reproductive technology that has reached to the grassroots for faster genetic improvement in dairy animals. For achieving great success through AI and for attaining the better results of AI outcomes, use of good quality semen is pivotal. Till date, for semen quality evaluation, semen volume, sperm concentration, and progressive motility are considered as gold standard methods although these parameters are insufficient and inefficient to distinguish a superior quality semen sample from an inferior one. Numerous studies have revealed different sperm functional attributes and their relation to fertility both in vitro or in vivo (Wiltbank and Parish 1986; Al-Makhzoomi et al. 2008; Karoui et al. 2012; Ahmad et al. 2003; Peña et al. 2009; Christensen et al. 2011). These parameters include plasma membrane integrity, acrosome integrity, capacitation status, mitochondrial membrane potential, apoptosis, chromatin integrity, reactive oxygen species, etc. (Singh et al. 2016; Kumaresan et al. 2017; Holden et al. 2017). The incorporation of a set of these parameters to the routine semen analysis would be more advantageous and assist us in the selection of superior quality of semen samples (Rodriguez-Martinez and Barth 2007).

Semen contains heterogeneous population of spermatozoa with varying degrees of structural and functional differentiation and normality, which results in different subpopulations of spermatozoa with each subpopulation varies in its fertilizing ability that differs within individual bull (Singh et al. 2016). To enrich semen samples with superior spermatozoa subpopulation, increasing sperm concentration per insemination dose is not a practical option as it also delivers inferior subpopulation resulting no significant improvement in sperm quality. Many studies have been carried out to sort and select the superior subpopulation of spermatozoa for better fertility outcomes. Whereas, purifying the given semen sample using suitable techniques such as washing, migration, filtration and differential gradient centrifugation offers better results in terms of removing dead or non-motile spermatozoa, reduces bacterial load and limits somatic cell contamination. The main drawbacks of these sperm purification techniques are low yield, variable efficiency (10–63%), time-consuming, and the sperm cells undergo stressful events such as centrifugation, which may deteriorate the vital sperm functional attributes (Morrell et al. 2010).

Recently, nanotechnology has emerged as an important tool that offers greater scopes in the area of semen biology. Using metal core nanoparticles conjugated with suitable antibodies against target proteins expressed in sperm surface membrane, superior quality spermatozoa can be effectively enriched in a given semen sample (Odhiambo et al. 2014). The advantage of using nanoparticles is that they have

magnetic properties and can be easily coated with suitable organic substances. The modified surface of nanoparticles can be functionally grafted using suitable biomolecules of our interest (Feugang 2017). Keeping this idea in mind, few studies have been conducted targeting important sperm functional parameters like acrosomal integrity, apoptotic status, abnormal spermatozoa (magnetic nanoparticles coated with fluorescent dye conjugated antibodies developed against specific marker proteins, i.e. anti-ubiquitin, FITC-PNA coated nanoparticles, etc.) following magnetic separation of undesired subpopulation of spermatozoa (Sutovsky and Kennedy 2013; Odhiambo et al. 2014; Durfey et al. 2017; Durfey et al. 2019). The resulting nano-enriched population showed significant improvement in semen quality parameters (Feugang 2017). In this chapter, different sperm subpopulations in a given ejaculate, purification of semen to enrich a particular subpopulation and different methods of semen purification are discussed with due emphasis to nano purification of semen.

6.2 Spermatozoa Subpopulation and Their Relationship with Fertility

Semen is a mixture of spermatozoa and seminal plasma. Not all the spermatozoa in a given ejaculate are similar; there exists different subpopulations because (1) spermatogenesis is a continuous process adding spermatozoa to already existing sperm pool (2) of the level of sperm maturation (although sperm epididymal maturation time is fixed for a species, individual sperm maturation duration differs based on the sperm output from the testis) and (3) of the frequency of ejaculation. It is shown that each subpopulation differs in their fertilizing potential (Sousa et al. 2011; Yániz et al. 2018; Singh et al. 2016; Ferraz et al. 2014). In order to accurately evaluate the relationship between fertility and sperm functional attributes, we need to evaluate sperm subpopulations having consistent relationship with fertility. For example, subpopulation of motile spermatozoa with intact membrane will have higher chance of fertilizing the oocyte in comparison to the membrane compromised spermatozoa. Similarly, many subpopulations exist in a given semen ejaculate such as live, dead, moribund, live acrosome intact, live acrosome reacted, high vs low mitochondrial membrane potential, apoptotic spermatozoa, etc. which can be clearly identified using high throughput technologies such as flow cytometry. For successful fertilization and embryonic development, sperm subpopulation with superior functional attributes and high fertilizing potential are highly desirable. Therefore, many studies have been conducted to enrich the semen sample with good subpopulation spermatozoa to obtain higher fidelity in AI, in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) outcomes (Correa et al. 1997; Samardzija et al. 2006; Muiño et al. 2008; Ferraz et al. 2014). In this line, a study by Morrell et al. (2011) showed beneficial effect in the motility of spermatozoa using sperm washing, migration and filtration techniques. Although these techniques were successful in enriching semen with high motile spermatozoa, they were unable to distinguish acrosome reacted, capacitated, apoptotic and loose chromatin

spermatozoa. Soon after, Morrell and coworkers in 2018 devised a semen purification technique based on single layer colloid centrifugation (SLC) which could enrich spermatozoa with higher chromatin compaction (low free thiols) and higher motility. The relationship of different spermatozoa subpopulation with bull fertility was shown in a study by Singh et al. (2016) where live acrosome intact sperm subpopulation was positively correlated with bull fertility but moribund, apoptotic, lipid peroxidized plasma membrane, necrotic subpopulations were negatively correlated with bull fertility. By considering suitable combinations of different subpopulations, Kumaresan et al. (2017) devised a fertility prediction model combining live, dead, live H_2O_2–ve spermatozoa and DFI with 83% accuracy. All these studies indicate the possibilities of obtaining higher conception rates with purified semen; the semen purification techniques are discussed below.

6.3 Semen Purification Techniques

Semen purification methods can be broadly classified into two, one is the removal of seminal plasma by means of centrifugation and/or washing and the other method aims to select spermatozoa based on certain functional attributes, such as sperm migration (based on sperm motility and/or membrane integrity) and colloid centrifugation (motility, morphology, viability and chromatin integrity). Colloid centrifugation can be further subdivided into density gradient centrifugation (DGC) and single layer centrifugation (SLC). In this book, a separate chapter is dedicated to colloid centrifugation process, results and its use in different assisted reproductive techniques. Basics of sperm purification techniques are discussed here.

6.3.1 Sperm Washing

Sperm washing is aimed at separating most of the seminal plasma from extended semen (Björndahl et al. 2005). The semen is centrifuged, and then the supernatant (contains most of seminal plasma and extender) is removed from spermatozoa (Fig. 6.1). The drawbacks of this technique are that superior subpopulation of spermatozoa cannot be selected and the washed semen sample contains undesired subpopulations such as abnormal, dead, necrotic, chromatin damaged spermatozoa (Hallap et al. 2004, 2000).

6.3.2 Sperm Migration/Swim-Up

Using suitable migration media, this technique utilizes motility parameters of spermatozoa to separate high-motile spermatozoa population from the low-motile spermatozoa. Swim-up is a simple method that is being routinely used to separate motile from non-motile spermatozoa in vitro (Magdanz et al. 2019). In this technique, swim-up media (e.g. modified Tyrode's media) is layered over sperm

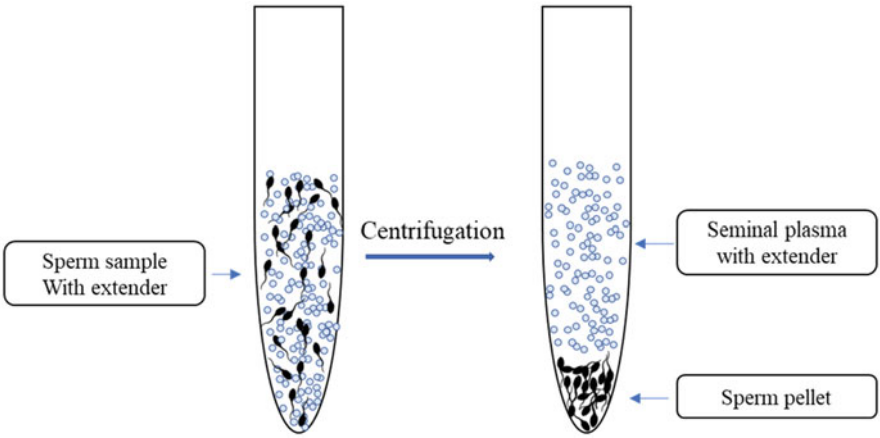

Fig. 6.1 Sperm washing using centrifugal force

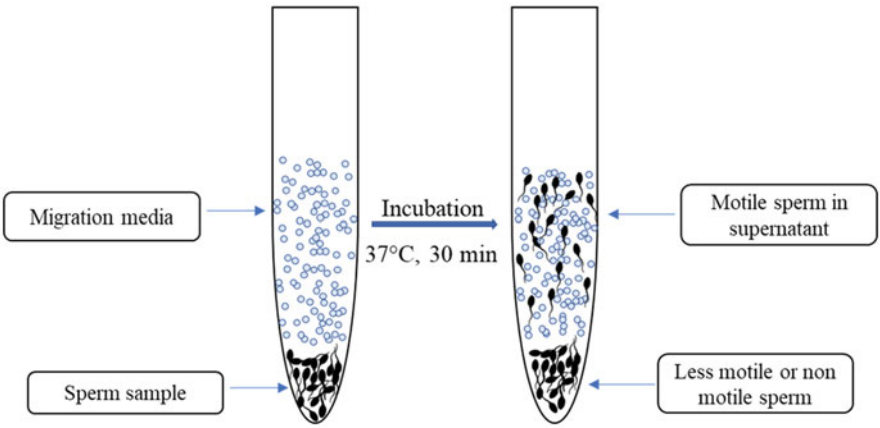

Fig. 6.2 Sperm swim-up

suspension and incubated at 37 °C. Those spermatozoa having motility, higher average velocity, higher percentage of normal morphology migrate towards upper fraction of swim-up media, whereas immotile and spermatozoa with tail abnormalities are prevented (due to their inherent inability to swim-up) from migration into swim-up medium (Fig. 6.2). Hallap et al. (2004) showed significantly improved midpiece and tail morphology after swim-up than after washing. Also, the swim-up method can become a more relevant model for mimicking the requirements for sperm travel in the female reproductive tract to reach the fertilization site. The resultant spermatozoa from upper fraction have shown to improve fertilization rates in vitro in mammals (Shittu et al. 2006; Grasa et al. 2004; Esteves

et al. 2000). However, the main disadvantage of these migration methods is the low recovery rate which ranges from 10 to 20% (Morrell et al. 2010).

6.3.3 Filtration

Glass fibres, Sephadex beads or membrane filters are generally used for selecting spermatozoa on the basis of their ability to move or plasma membrane intactness (Fig. 6.3) (Mogas et al. 1998). Non-viable or membrane compromised spermatozoa adhere to the matrix more effectively than motile and membrane intact spermatozoa although the mechanism behind this is still unknown. Using filtration methods, few studies (Mogas et al. 1998; Januskauskas et al. 2005) on canine and bovine species showed improvement in post-thaw sperm viability and decreased population of acrosome reacted spermatozoa. Many studies have reported that spermatozoa filtration is useful in eliminating leukocyte contamination (possibly due to their tendency to adhere to filter coupled with less mobility) and selecting morphologically normal spermatozoa. Although filtration cannot eliminate all seminal plasma and debris from the given semen sample, approximately 63% recovery rate has been reported (Morrell et al. 2010).

6.3.4 Colloid Centrifugation

Colloid centrifugation of semen implies centrifugation of extended semen through a colloid, targeting separation of seminal plasma and enriching the motile, viable and chromatin intact spermatozoa. In colloid centrifugation method, density gradient centrifugation (DGC) is one of the methods that has been recommended by the World Health Organization for human spermatozoa purification to be used for

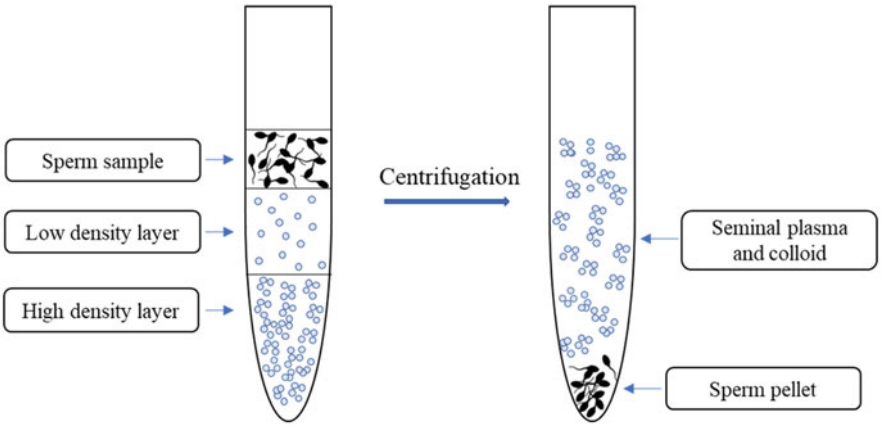

Fig. 6.3 Density gradient centrifugation (DGC)

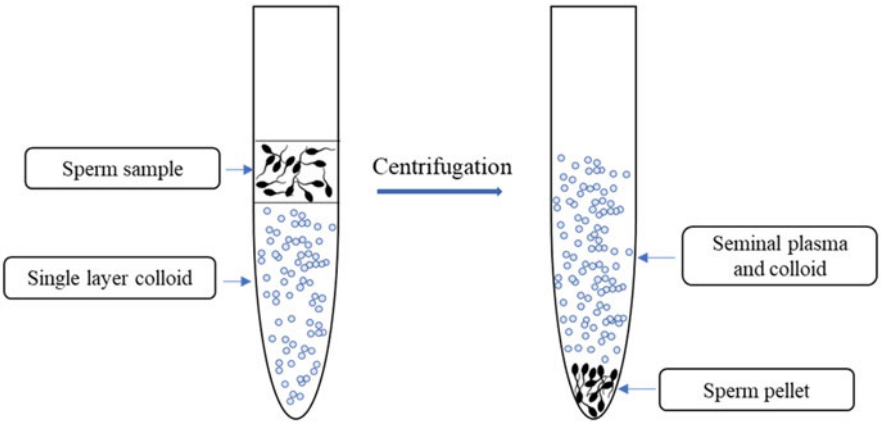

Fig. 6.4 Single layer centrifugation (SLC)

various assisted reproductive techniques. Of late, this method has been suggested as a better alternative to simple washing to improve the attributes of animal sperm preparations (Morrell and Rodriguez-Martinez 2009; Rodriguez-Martinez et al. 1997). In density gradient centrifugation technique, sperm selection is based on application of density gradient to separate debris, epithelial cells, egg yolk particles, immotile spermatozoa from motile sperm population, thus ensuring presence of high-motile spermatozoa in purified sample with adequate fertilizing potential. The most commonly method used for gradient sperm selection in bovine IVF laboratories in a large scale is Percoll®, a colloidal solution containing non-dialyzable polyvinylpyrrolidone (PVP), that does not affect the osmotic pressure of the medium and is stable at a wide range of pH 5–10. Centrifugation with Percoll® separates spermatozoa based on their plasma membrane integrity, and eliminates other cells, detritus and bacteria (Oshio 1988). In this procedure, the sperm sample is placed on the top of a higher density Percoll medium and then centrifugated. The pellet at the bottom is thus enriched with high-quality spermatozoa after the most active spermatozoa population with higher motility penetrate the boundary, therefore, successfully separating them from debris, sperm fragments and immotile sperm (Fig. 6.4). Although this method provides a higher recovery of motile spermatozoa and the sperm quality is higher as compared to simple washing technique, it requires additional centrifugation which may result in drastic changes in plasma membrane, acrosome leading to decreased sperm functionality (Somfai et al. 2002). However, this method is quick and one of the most effective methods for removing seminal plasma and extenders used during cryopreservation (Lee et al. 2009).

One of the recent modifications of existing DGC method is single layer centrifugation (SLC), where semen is layered on top of a layer of colloid, thus nullifying the need for preparation and layering of several colloids having different densities. Following centrifugation, the seminal plasma remains on top of the colloid layer and spermatozoa migrate towards the bottom of centrifuge tube due to centrifugal

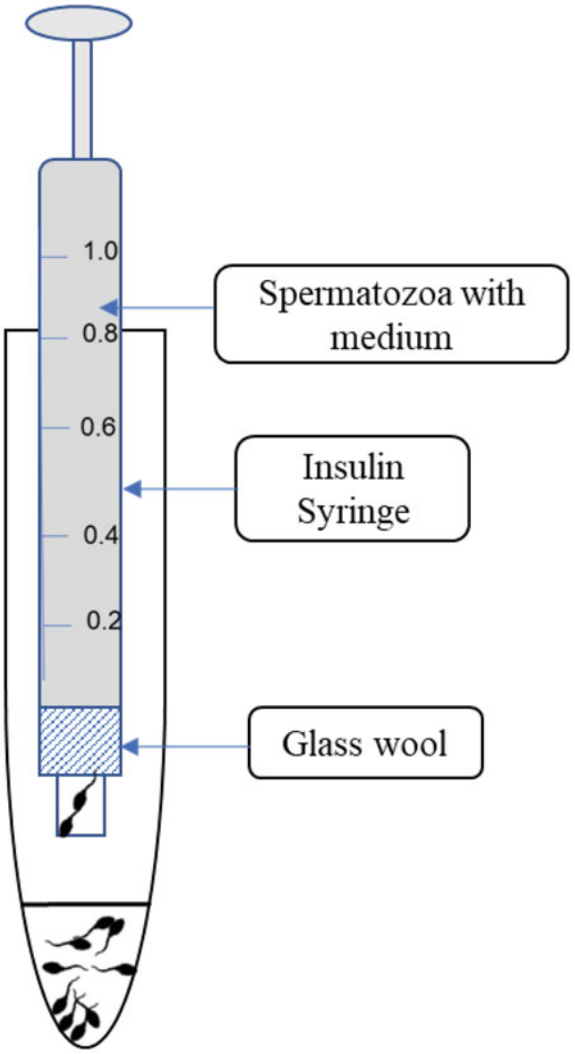

Fig. 6.5 Glass wool filtration

force (Fig. 6.5). SLC method has shown to select good quality spermatozoa without damage to plasma membrane, DNA and acrosome. Also, the volume of semen that can be purified using this method is reasonably higher as compared to DGC method allowing this method to be scaled-up to process a large number and volume of ejaculates (Morrell et al. 2011). Interestingly, it has been found that SLC technique significantly minimizes pathogenic load in semen, thus can potentially increase the longevity of spermatozoa in ejaculate (Morrell and Wallgren 2011). In stallions and boars, SLC proved to be beneficial in maintaining viability and chromosome integrity as compared to sperm washing (Morrell et al. 2010). Being a potential means to select robust spermatozoa population from ejaculate, SLC has shown to select

metabolically active and morphologically normal spermatozoa in bull ejaculate (Nongbua et al. 2017). The overview of sperm purification techniques is presented in Fig. 6.6.

6.4 Nano Purification

Nanotechnology refers to research, production and use of nanoparticles (1–100 nm) at the molecular level. Synthesized nanoparticles are versatile in nature and have shown multifunctional applications in biomedical research. Nanoparticles can be obtained from various sources such as carbon based, metals and their oxides, various polymers and semiconductors. However, among these, iron oxide nanoparticles particularly magnetite (Fe_3O_4) are attracting the researchers worldwide due to their better magnetic properties as well as higher degree of bioavailability and safety (Fig. 6.7). Recently, nanoparticles are being used in the field of human as well as animal andrology to separate subpopulation of spermatozoa with aberrant phenotypes from a given semen sample, and the nanopurified semen showed beneficial effects in phenotypes and other functional attributes. Due to widespread applications of iron magnetic nanoparticles in various aspects of reproductive medicine, preparation, characterization and applications of the same are discussed in the following sections.

6.4.1 Synthesis and Characterization of Nanoparticles

The principle behind production of iron magnetic nanoparticle is to coat the particles with suitable antibodies or lectins for targeted capture and removal of desired or undesired cell populations. Several techniques are available for production of iron magnetic nanoparticles with appropriate surface chemistry, i.e. physical methods, chemical methods and biological methods. In physical method, deposition of gas phase or electron beam lithography is carried out for production of magnetic nanoparticles (MNPs). However, there is no control on the size of produced MNPs and particles are either irregular spheres or rods (Cuenya 2010; Lin and Samia 2006). In biological method, microbial incubation results in production of small spherical or irregular rod shaped MNPs (Narayanan and Sakthivel 2010). Recently, chemical-based methods are adopted in a large scale due to their controlled size production, lower cost of production and higher yield (Ali et al. 2016). Several techniques are available in chemical method of MNP production, namely sol-gel methods, oxidation, hydrothermal, chemical co-precipitation, flow injection, etc. (Laurent et al. 2008; Wu et al. 2011; Woo et al. 2004; Salazar-Alvarez et al. 2006).

The most commonly used chemical method is chemical co-precipitation method where MNPs are synthesized by adding a base to an aqueous mixture of Fe^{2+} and Fe^{3+} chloride at a molar ratio of 1:2, resulting precipitation of black iron nanoparticles. An oxygen-free environment is required to prevent iron nanoparticles from either oxidation or agglomeration or both. Iron nanoparticles are usually coated

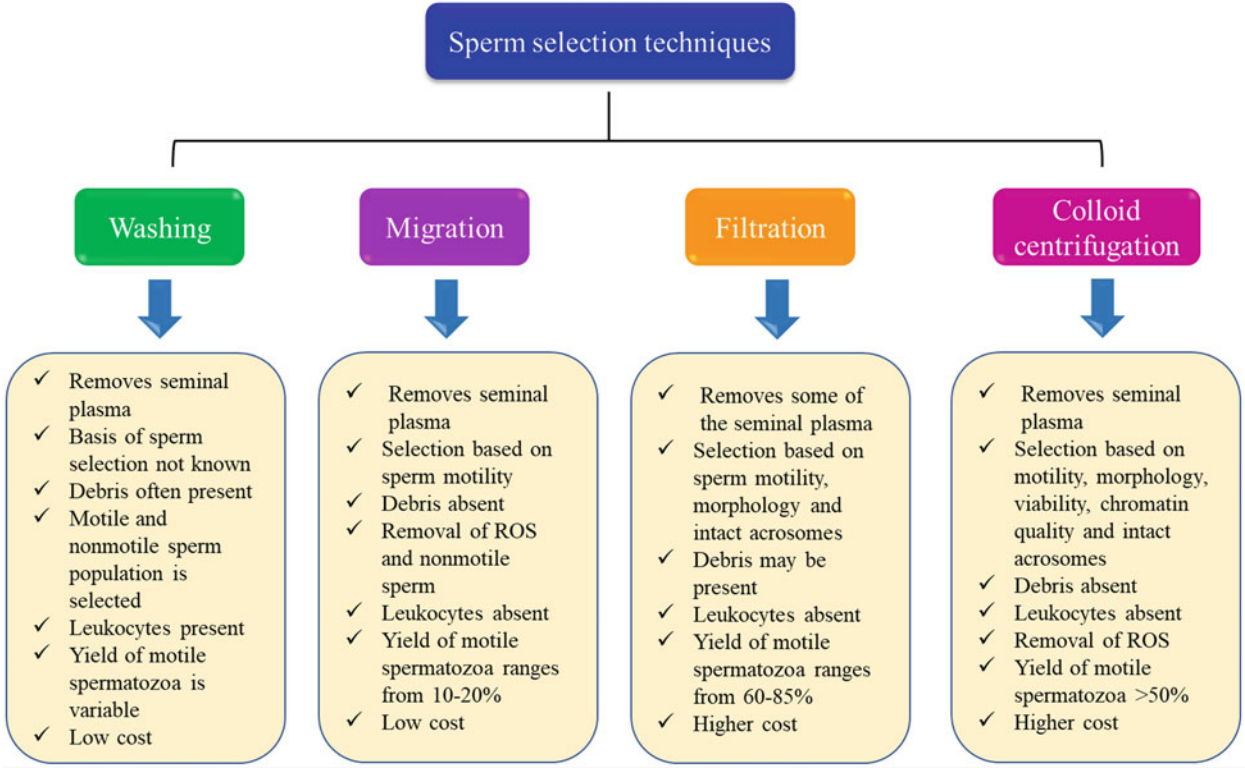

Fig. 6.6 Overview of sperm selection techniques

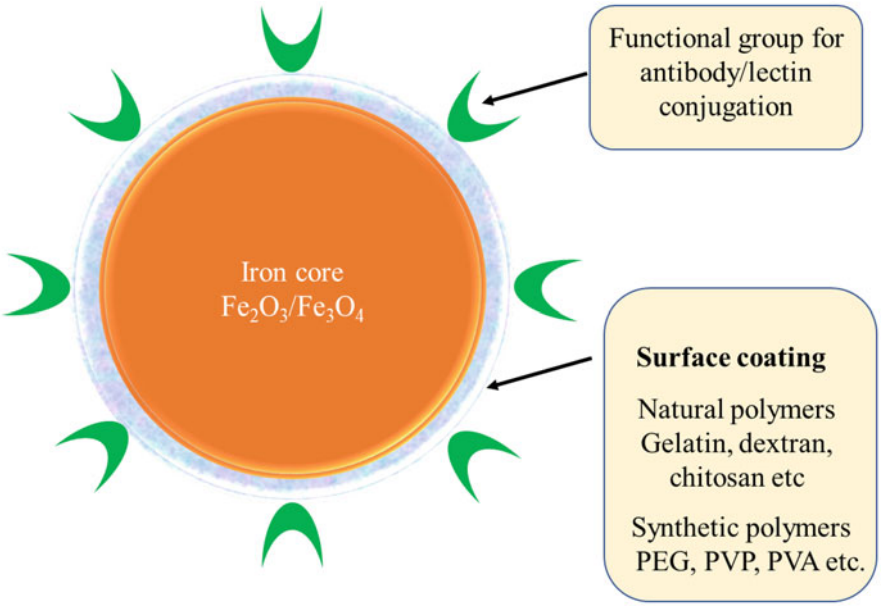

Fig. 6.7 Basic structure of iron magnetic nanoparticle

with organic or inorganic molecules (e.g. silica, dextran, chitosan, etc.). However, it is a prerequisite to synthesize MNPs in oxygen-free environment, preferably in presence of N_2 gas. Bubbling of N_2 gas not only prevents nanoparticle oxidation but also reduces its size (Maity and Agrawal 2007). A complete precipitation of magnetite is likely to occur between pH 9 and 14 by using alkaline solutions like ammonia or sodium hydroxide.

The formed nanoparticles are characterized for structure, morphology and its magnetic properties and for dextran functionalization. Commonly, dynamic light scattering (DLS) (size distribution based on hydrodynamic), transmission electron microscope (TEM) (shape heterogeneity, size navigation), scanning electron microscope (SEM) (size distribution), X-ray diffraction (XRD) (size, shape and structure determination of crystalline structures), Fourier transform infrared spectroscopy (FTIR) (surface properties such as polymer functionalization and conformation) are used for nanoparticle characterization (Ali et al. 2016). The workflow of production of nanoparticles and their characterization is described in Fig. 6.8.

6.5 Application of Nanotechnology in Reproductive Biomedicine

The field of reproductive biology is constantly growing in understanding the complex processes of gametogenesis, fertilization, implantation as well as embryonic growth and development in both humans and other mammals. The success in

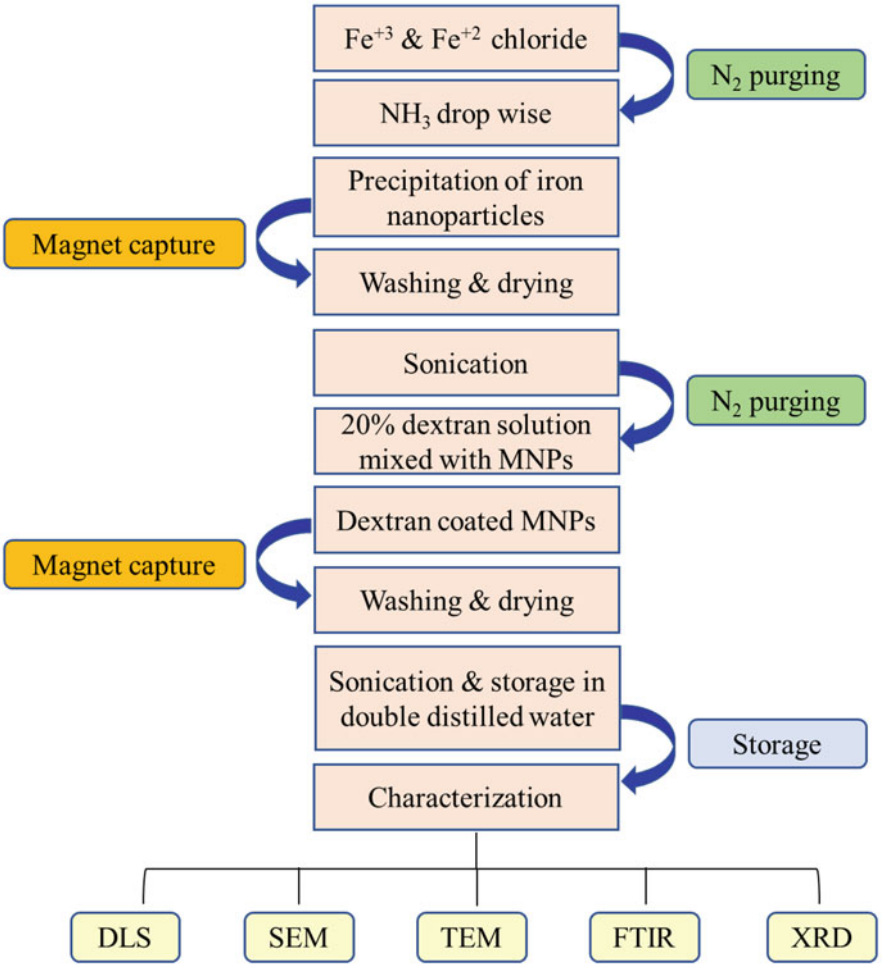

Fig. 6.8 Overall view of preparation & characterization of iron magnetic nanoparticles

fertilization is chiefly based on the fertilizing potential of gametes and in this regard both sperm and oocyte are equally important. Nanoparticles based on their size, shape, core material are widely used in various aspects of reproductive medicine and assisted reproduction. As nanoparticles can be prepared in a controlled manner to produce particles with desirable size and shape, they are a suitable carrier for targeted delivery of drugs and other biomolecules to exert their function. Due to their relatively small size, nanoparticles can be easily endocytosed in target cells as well as can travel various intercellular compartments. Thus, nanoparticles can be used in various reproductive affections, drug delivery, gene therapy as well as Magnetic-Activated Cell Sorting (MACS) (Doroftei et al. 2015).

6.6 Magnetic Nanoparticle (MNP) Based Semen Enrichment for Superior Spermatozoa

One of the important applications of nanotechnology is improving semen quality using nano purification technique. There is a growing interest in magnetic nanoparticle-based semen enrichment techniques due to their ease in production, novel magnetic nature as well minimal detrimental effects on the sperm quality. A simplified protocol of sperm nano purification is depicted in Figs. 6.9, 6.10.

Since many years/for the past decades, the application of nanoparticle-based semen selection has been attempted in several species and found to be a potential alternative to already existing semen purification techniques (Odhiambo et al. 2014; Durfey et al. 2019; Yousef et al. 2020). Odhiambo et al. (2014) reported that using PNA coated nanoparticle, purification of bull spermatozoa was carried out and upon filed trial AI on 798 cows, a conception rate of 64.5 ± 3.7% was obtained with ten million nano purified sperm dose. When non-purified dose of ten million was used, there was significant decrease in conception rate (53.7 ± 3.2%). The main advantages of semen nano purification were higher average sperm recovery rate (70%), and offspring produced after AI with nano purified spermatozoa were normal in terms of fertility (normal pregnancy outcomes in heifers).

A similar study has been conducted in boar semen using double nano purification procedure to eliminate acrosome reacted as well as apoptotic subpopulation of spermatozoa (Durfey et al. 2019). MNPs conjugated with FITC-PNA remove acrosome reacted spermatozoa in first nano purification, whereas Annexin V coated MNPs were used for the second nano purification. Thus, using Fe-magnetic nanoparticles coated with fluorescent conjugated Annexin V and PNA, acrosome reacted and apoptotic subpopulations can be successfully eliminated. In this line, Yousef et al. (2020) conducted PNA coated MNP (surface coating was done using chitosan) based semen purification of donkey spermatozoa and found improved post-thaw sperm motility and acrosome integrity. The plasma membrane integrity as well as sperm morphology remained unaltered after nano purification procedure. This method also yielded sperm recovery rate of approximately 80%. They further suggested that semen nano purification can be an effective tool in reducing proportion of acrosome reacted spermatozoa in frozen semen and thus can improve quality of frozen thawed donkey spermatozoa.

Although studies on semen nano purification in bull spermatozoa are scanty, in buffaloes this method has shown to improve the quality of raw semen by trapping the dead and damaged spermatozoa in magnetic nanoparticles. Bisla et al. (2020) have shown that ubiquitin coated iron magnetic nanoparticles were effective in improving post-thaw motility as well as DNA integrity in spermatozoa as compared to non-purified semen. Also, the total antioxidant capacity and superoxide dismutase activity were found to be significantly higher in nano purified semen as compared to control. Currently, we are also working on dextran coated iron magnetic nanoparticle-based bull sperm enrichment by selectively eliminating acrosome reacted sperm subpopulations and are obtaining encouraging results (unpublished data; Fig. 6.11).

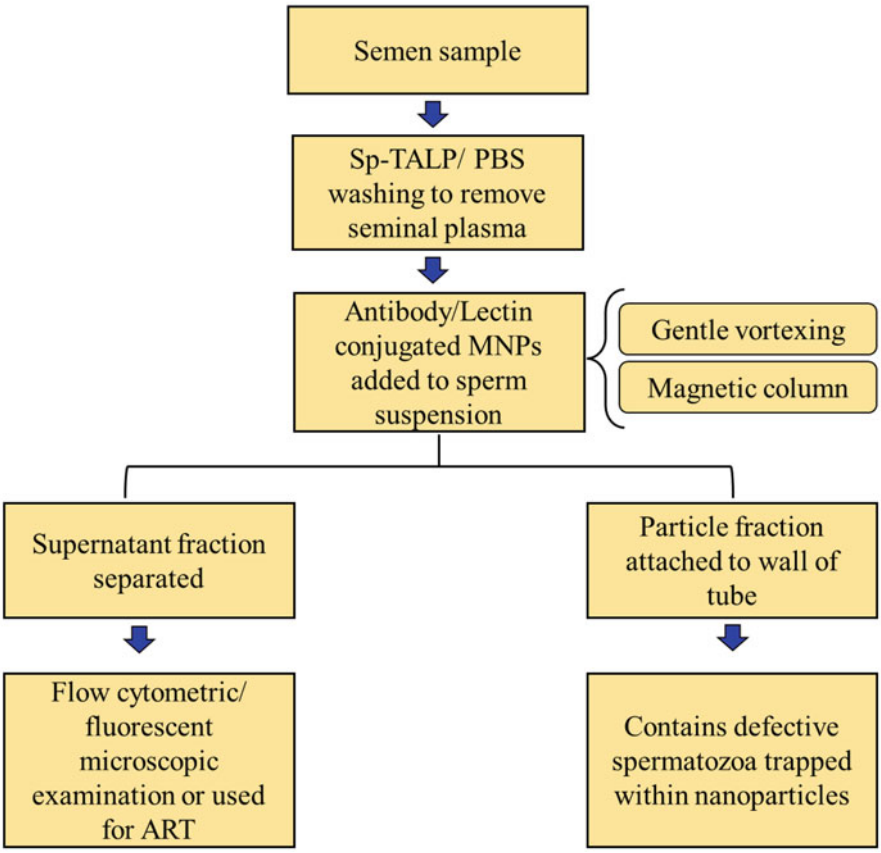

Fig. 6.9 Semen nano purification protocol

All these above-mentioned studies indicate that magnetic nano purification offers us a greater scope in future to include many more fertility related proteins/lectins in the panel of biomarkers so that we can come with an efficient nano purification procedure that will enhance the sperm functional competency. Some of the fertility associated proteins, which can be targeted using magnetic nano purification technique are presented in Table 6.1.

6.7 Negative Biomarker-Based Sperm Selection

Negative biomarker-based approach to andrological evaluation primarily focuses on proteins or lectin ligands that differentially or exclusively present on the surfaces of abnormal or damaged spermatozoa. The term "negative" is used because these biomarkers are negatively related to sperm functional attributes and results in

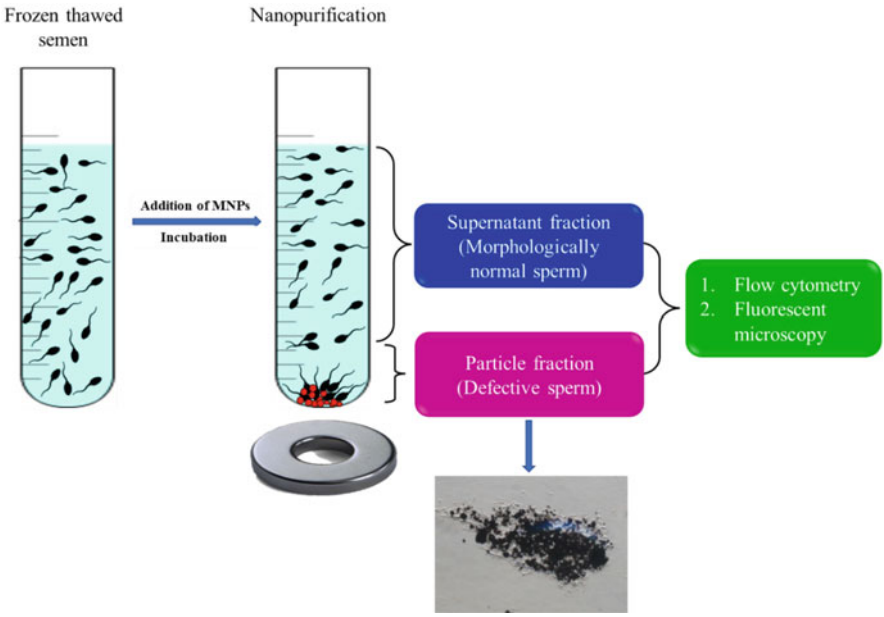

Fig. 6.10 Basic procedure for semen nano purification

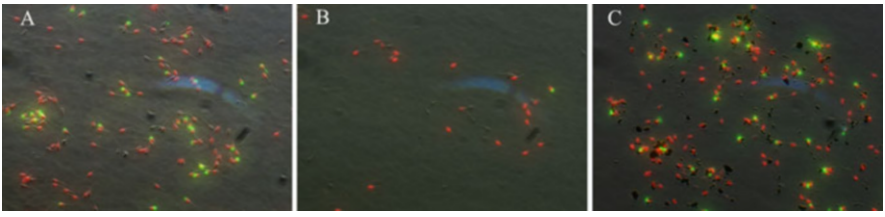

Fig. 6.11 Semen nano purification using FITC-PNA conjugated to dextran coated iron magnetic nanoparticles. (**a**) FITC-PNA + PI staining of non-purified sperm sample, (**b**) FITC-PNA + PI staining of nanopurified sperm sample, (**c**) FITC-PNA + PI staining of particle fraction containing sperm bound to iron nanoparticles. All images were visualized under 200X magnification (unpublished data from the authors)

reduced fertility or infertility (Sutovsky et al. 2015). In the field of human andrology, few markers for fertility have been identified. One such example is spermatid specific thioredoxin SPTRX3 which is retained in residual cytoplasm of defective human spermatozoa. Semen samples having higher SPTRX3 have shown to increase fertilization failure following assisted reproductive therapies (ART) in human and are more prone to recurrent miscarriage. Some other negative markers for sperm quality and fertilizing potential have been identified in human as well as in other species which will be discussed in the subsequent sections. Incorporation of these marker proteins or lectins in nano purification has a tremendous potential for quality

Table 6.1 Sperm fertility associated proteins that can be targeted using magnetic nano purification technique

Sl no	Name	Localization	Marker for fertility	Reference
1	PAWP	Post-acrosomal sheath (PAS) of the spermatid	Positive	Sutovsky and Oko (2011)
2	HBP 30	Seminal plasma, sperm membrane	Positive	Bellin et al. (1998)
3	Ubiquitin	Plasma membrane	Negative	Sutovsky (2003)
4	Clusterin	Bull reproductive tract fluid, plasma membrane	Negative	Ibrahim et al. (1999)
5	PAFr	Plasma membrane	Negative	Roudebush and Diehl (2001)

sperm enrichment (from bulls with higher genetic merit but donating poor quality ejaculate due to various conditions).

6.7.1 Ubiquitin

Ubiquitin is a small (76 amino acid residues) proteolysis-promotingpost-translational protein modifier. Ubiquitin is an important member in ubiquitin-proteasome system (UPS) (Glickman and Ciechanover 2002). Ubiquitin and other components of UPS are secreted by the principal epididymal epithelial cells in an apocrine fashion (apical blebs). Defective spermatozoa become ubiquitinated during their epididymal transit and selectively eliminated due to intervention of chief constituents of UPS (Sutovsky et al. 2001; Paul et al. 2021). Ubiquitinated bull spermatozoa display increased sperm DNA fragmentation (Sutovsky et al. 2002. Sperm surface ubiquitination has negative effects on sperm count, motility, morphology and increases the incidence of acrosomal damage and higher conception failure after AI (Sutovsky et al. 2002; Kennedy et al. 2014; Odhiambo et al. 2011). Thus, ubiquitin can be considered as a useful marker to distinguish semen using anti-ubiquitin antibodies, which might be beneficial in eliminating abnormal spermatozoa from morphologically normal and functionally competent spermatozoa in a given semen sample.

6.7.2 Lectins

Fluorescently labeled lectins (plant proteins) have the ability to sense and couple with glycosidic residues in different parts of acrosomal membrane (Odhiambo et al. 2011). In a normal acrosome, the ligands for lectins are not expressed or precisely buried underneath, but an abnormal/acrosome reacted spermatozoa expresses ligands for those lectins. One such example is lectin PNA (peanut agglutinin isolated from the plant *Arachis hypogea*), which is the most commonly used lectins because of their specificity. Only spermatozoa with reacted, abnormally formed or damaged

acrosome participate in biding with lectins and emit green fluorescence (FITC fluorophore-conjugated PNA) which can be visualized under microscope or in flow cytometry (Graham et al. 1990; Nagy et al. 2003). Similar lectins are also available such as PSA (*Pisum sativum* agglutinin) and LCA (*Lens culinaris* agglutinin) though their specificity is lower as compared to PNA (Sutovsky and Kennedy 2013).

6.7.3 Annexins

In mammalian cells, apoptosis is a physiological event characterized by programmed cell death without any inflammatory changes to the surrounding microenvironment (Wyllie et al. 1980; Martin et al. 2004). At the end of apoptosis, the nucleus undergoes significant fragmentation, and the cell forms blebs and fragments known as apoptotic bodies. During the early stages of apoptosis, there is loss of plasma membrane asymmetry of cells resulting externalization of phosphatidylserine (PS) on the outer leaflet of membrane (in normal and healthy cells, the PS is present on the inner leaflet of plasma membrane). The externalization of PS tags the apoptotic cells to be identified and later phagocytosed by surrounding healthy cells (Anzar et al. 2002). Like other mammalian cells, spermatozoa in male reproductive system undergoes programmed cell death and later are eliminated by means of phagocytosis. However, failure to remove apoptotic (defective) cells can lead to high incidence of abnormal sperm in semen and low fertility (Yin et al. 1998).

Identification of apoptosis in spermatozoa can be carried out using a flowcytometric approach based on staining cells with annexin and propidium iodide (FITC-Annexin V+ PI). Annexin V is a Ca^{+2}-dependent, phospholipid-binding protein (35–36 kDa) having higher affinity for PS and binds to cells with externalized PS (Chaveiro et al. 2007). Annexin V conjugated to fluorescein isothiocyanate (FITC) fluorochrome retains its high affinity for PS in a calcium rich environment and, therefore, serves as a sensitive probe to be used for flow cytometric detection of cell death. Annexin V in combination with PI aids in detecting apoptotic and necrotic spermatozoa from viable spermatozoa population when analysed using flow cytometry or fluorescent microscopy.

Therefore, a suitable tool to combine ubiquitin, lectins and annexin together for combo elimination of abnormal, damaged and acrosome reacted, and apoptotic spermatozoa can form a basis to enrich a given sample of semen with morphologically normal and superior quality spermatozoa.

6.8 Safety of Using Iron Nanoparticles in Semen Nano Purification

Surface coating of iron nanoparticles using dextran or other polymers has shown to minimize aggregation and cytotoxicity of MNPs (Lind et al. 2002). In this regard, size of nanoparticles plays an important role in determining the cytotoxicity of

MNPs. In general, it has been found that cytotoxicity increases with particle size (Yin et al. 2005; Gorth et al. 2011). However, such kind of toxicity study of nanoparticles on sperm cells are not available in a detailed manner. Durfey et al. (2017) conducted nano purification of boar spermatozoa using PNA and Annexin coated nanoparticles and found that the sperm quality was improved after nano purification. Also, upon insemination using nano purified fraction of spermatozoa, the resultant offspring showed normal postnatal health, growth and development parameters. Recently, we also have assessed the effect of dextran coated iron superparamagnetic nanoparticles on post-thaw semen quality in bulls and found that dextran coated nanoparticles did not show any detrimental effect on viability, acrosome integrity, mitochondrial ROS production and mitochondrial membrane potential in a time dependent manner, thus suggesting protective effects of dextran coating on spermatozoa.

6.9 Perspective and Prospective

Enrichment of a given semen sample with spermatozoa having desired characteristics, functionalities and fertility has been the demand of livestock breeders since long time. Sperm washing and sorting of spermatozoa using their migration ability are the methods commonly employed in selection of spermatozoa. Thereafter, sperm filtration-based techniques gained momentum; however, low recovery rate and inability to track functionally inferior spermatozoa limits their usage in large scale. Colloidal centrifugation technique resulted in selection of good quality morphologically normal and metabolically active spermatozoa with intact plasma membrane, DNA and acrosome. The technique of sperm separation by magnetic activation (MACS) is a non-invasive method that uses knowledge of conjugating specific molecules that has the affinity to the sperm that are to be removed. When the mixture is exposed to a magnetic field, the target cells adhere to the magnetic column while the normal cells remain in the free suspension. It has been shown that nanoparticle-based sperm purification results in high recovery rates, which is an advantage of this technique over the others. However, the effect of nanoparticles on sperm functions and physiology needs to be assessed in detail. Any future developments in sperm purification/selection technique should ensure that technique that does not interfere with sample quality and allow for the production of high-quality semen samples. This will contribute to the increase in reproductive efficiency in the bovines.

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Sperm Transcriptome Sequencing for Predicting Bull Fertility: Concepts, Facts and Future Directions

7

S. Selvaraju, L. Ramya, D. Swathi, S. Parthipan, S. S. Archana, B. K. Binsila, B. Krishnappa, and A. Arangasamy

Abstract

Composition of sperm biomolecules and their function in domestic animals has not been detailed yet. Unlike somatic cells, sperm contains low quantity of biologically degraded RNAs, extraction and profiling of total RNA from sperm become challenging to the reproductive biologists. Sperm RNAs are extracted using cocktail of lysis solution and successfully profiled employing various RNA sequencing platforms. Bioinformatic pipeline has been standardized for the analysis of sperm transcriptome data. Emerging evidences suggest that sperm RNAs can be of potential markers to assess the spermatogenic events and success of the fertilization process. Sperm RNAs are also potential candidates to predict successful embryonic development, pregnancy maintenance as well as the health and well-being of the offspring. In human sperm, RNA elements are reported to have diagnostic ability in predicting the success of assisted reproductive technologies. Thus, sperm RNA profiling can be a non-invasive method for predicting fertility of bulls. In future, this may become an initial step for screening bulls for breeding program.

Keywords

Sperm RNA · RNA sequencing · Bioinformatics · Spermatogenesis · Fertility

S. Selvaraju (✉) · L. Ramya · D. Swathi · S. Parthipan · S. S. Archana · B. K. Binsila · B. Krishnappa · A. Arangasamy
Reproductive Physiology Laboratory, Animal Physiology Division, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka, India
e-mail: Selvaraju.S@icar.gov.in

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Sexual reproduction is a complex process involving integration of male and female gametes' genome to form an embryo. Successful development of the foetus and birth of an offspring depends on the molecular composition and functional ability of the gametes. In mammals, fertility is declining over a period of time, and the reduced fertility rate could be attributed to male, female, and other factors. In the bovine breeding industry, wherein artificial insemination (AI) is widely practiced, the role of bulls becomes utmost important, because semen samples collected from one bull are used to inseminate thousands of cows. The bulls are selected for the AI program based on the minimum standard protocol, which includes evaluation of phenotype, quantitative trait loci (QTL), single nucleotide polymorphisms (SNP), dam and daughter's milk yield and conventional semen analysis including mass activity, concentration and motility (Binsila et al. 2017). Recently, the fertility status of the bulls is ascertained by assessing the seminal and the sperm functional attributes such as the plasmalemma integrity, sperm motility, functional membrane integrity, acrosome integrity and mitochondrial membrane potential (Selvaraju et al. 2013). In spite of these set criteria, the cattle and buffalo conception rate at the field is only 30–45% Animals at par with the seminal attributes are identified to have varied conception rates, and this indicates that spermatozoal genetic composition has an important role in deciding its competency for successful pregnancy outcomes.

Sperm is destined to meet the oocyte and deliver its biomolecular contents to produce a healthy viable offspring (Jodar et al. 2015). The genetic content comprises of biomolecules such as DNA, RNAs, proteins and metabolites. With the known roles of DNA, proteins and metabolites in any biological process, the presence of spermatozoal RNAs in a transcriptionally silent cell is startling and provoking to study the sperm transcriptome and understand its function on the peri-fertilization. Recent studies suggest that the transcripts retained in the sperm are not random and provide complete information from spermatogenesis to the successful birth and viability of the offspring (Jodar 2019; Selvaraju et al. 2018). Hence, this chapter is focused on the recent developments in sperm transcriptome profiling for understanding the functional associations of sperm transcripts with fertilization process.

7.1 Why Do Sperm Carry RNA?

Sperm are transcriptionally and translationally silent. Sperm are structurally designed to deliver the haploid genome to an egg. The low success rate with respect to embryo development and birth of the offspring in somatic cell cloned and parthenogenetic experiments necessitated the researchers to elucidate the role of males on peri-fertilization events. For the past two decades, the reproductive biotechnologists are trying to explore the composition and functional role of various biomolecules including RNA, proteins and metabolites in the sperm. Earlier studies though suggested that the sperm RNA are remnants of spermatogenesis (Yang et al. 2009), recent research evidenced the composition of the sperm RNA and their importance in the fertilization process (Ostermeier et al. 2004; Selvaraju et al. 2017). Sperm carry both intact and fragmented mRNA along with other

non-coding RNAs such as small RNAs, long non-coding RNAs, interference RNA and retained RNA elements (Sendler et al. 2013; Bukowska et al. 2013). Studies proved that sperm RNAs can be used to assess the past events associated with spermatogenesis (Yadav and Kotaja et al. 2014), sperm function (Savadi-Shiraz et al. 2014; Tiwari et al. 2008) as well as the future processes such as fertilization (Lalancette et al. 2008), successful pregnancy (Arangasamy et al. 2011; Parthipan et al. 2017) and even health of an offspring (Jodar et al. 2015).

7.2 Verify, Contest and Accept

Sperm triggers the oocyte to proceed for final maturation to start the actual fertilization process and embryogenesis. Once the oocyte genome is activated, it starts verifying the sperm biomolecules entering the cytoplasm, contests and finally forms syngamy (Bourchis and Voinnet 2010; Miller 2015). During this process, paternally derived imprinted messages are recognized by the maternal genome and inherited to the next generations. Various classes of sperm-borne RNAs regulate these events of recognition and consolidation, which are essential for successful early embryogenesis (Jodar et al. 2013). During the production of interspecies hybrid offspring, failure of recognition and consolidation mechanisms lead to overexpression of retrotransposons and subsequent infertility (Miller 2015). For example, while crossing the cattle and buffalo, though fertilization is successful, embryo death occurs before or during the embryo genome activation. The same may also be responsible for the birth of sterile hybrid offspring in the case of a mule. In addition, placental abnormalities were reported while developing somatic cell cloned foetus. This could probably be due to absence of sperm-derived factors that are essential for nuclear reprogramming and cytoskeleton remodelling during somatic cell cloned embryo development, pregnancy maintenance and birth of the offspring. These findings emphasize the importance of spermatozoal RNAs in fertilization and successful pregnancy outcomes.

7.3 Importance of Whole Transcriptome Profiling for Sperm

Sperm are highly differentiated cells destined for fertilization, and their structural and functional features need to be considered before attempting to analyse biomolecules within them (Amaral et al. 2016). Unlike somatic cells, sperm has reduced cytoplasm, condensed nucleus (Johnson et al. 2011a, b), impaired ribosomal assembly (Cappallo-Obermann et al. 2011), low amount of RNA (Boerke et al. 2007) with biologically fragmented nature (Johnson et al. 2011a, 2011b). So technically challenging steps are introduced while extraction and sequencing of sperm RNA.

Routinely used RNA-quantification methods such as RT-PCR, microarray and other hybridization techniques have been superseded by the next-generation sequencing technology. The microarray techniques require good quality and

quantity of RNA, which cannot be met in the case of sperm RNA, which is inherently fragmented. In addition, microarray provides information only about the known genes. The study of transcriptome enables us to understand the functionality of the genes (both known and unknown) in various types of cells and tissues even with limited knowledge on the genome of the organism. Whole transcriptome profiling by next-generation sequencing techniques have been standardized for sperm with specific library preparation protocols to sequence both coding and the non-coding RNAs (Sendler et al. 2013). Transcriptome sequencing generates a huge amount of data, which requires an intense analysis for an appropriate interpretation (Ramya et al. 2021). The bioinformatics analysis ascertains the possible functional role of sperm RNA in various biological processes, a constituent of cellular component and molecular functions with enrichment functions and pathways that are majorly regulated in the spermatozoa. This can also be used to identify SNPs, QTLs and splice variants in sperm transcripts. Recently, single-cell RNA sequencing is in trend to allow the discovery and investigation of many cellular subtypes. Sperm are challenging cell type for the investigation of RNA at the single-cell level, as they differ from typical somatic cells in several aspects. However, studies are coming up employing single-cell RNA sequencing for sperm transcriptome (Tomoiaga et al. 2020). Exploring the single-cell transcriptome might be helpful to understand the underlying mechanisms that are responsible for sperm functional competence.

7.4 Isolation of Sperm RNA

The sperm contains highly condensed chromatin, very less cytoplasm and a limited amount of RNA (Johnson et al. 2011a, b). The structure and composition of sperm vary among species, and a unique RNA isolation method is necessary for each species as compared to other cells. Various methods such as Trizol, cold TRIZol, Hot TRIZol and membrane-based commercial kits were employed for the isolation of spermatozoal RNA (Bissonnette et al. 2009). However, the method developed in our laboratory (Parthipan et al. 2015) by modifying the existing protocol being used for the human sperm RNA isolation method (Goodrich et al. 2007) suits best for isolating the bull spermatozoal RNA

Some of the important considerations (Parthipan et al. 2015) while extracting sperm RNA are as follows:

1. Density gradient purification is essential to remove contaminating cells (somatic cells, germ cells and leukocytes) in the semen. This step is important as the somatic cells contain 200 times more RNA content than sperm.
2. The optimum bovine sperm input concentration should be 30–40 million per extraction. Higher concentration of cells may block the silica membrane resulting in poor yield and also more chances of gDNA contamination.
3. The cocktail of lysis solutions (TRIzol + lysis buffer provided in the kit) and phase separation step are important for the complete lysis and removal of

contaminating gDNA, respectively. An addition of 2-mercaptoethanol (1%) to the kit lysis buffer will help in the elimination of the RNases present.
4. The extracted total RNA invariably contains higher concentration of gDNA and should be treated with DNase.
5. Fluorometry as compared to the spectrophotometer should be used for accurate quantification of RNA. It is also to be noted that even fluorescent dye like RiboGreen does not differentiate intact RNA from free nucleotides (Jones et al. 1998).
6. The quality of RNA should be checked using spectrophotometer by considering 260/280 and 260/230 ratios to assess the protein and other solvent contamination, respectively. Bio-analyzer profiles should be carried out for evaluating the fragment size distribution and also to get an information about total RNA intactness.
7. An intron spanning primer should be used to check the presence or absence of gDNA contamination.
8. The extracted total RNA must be checked for other cell contaminations with the cell-specific primers for leukocytes (protein tyrosine phosphatase receptor type, C; PTPRC), somatic cells (Cadherin 1; CDH1) and germ cells (Kit oncogene; KIT) using real-time PCR.

7.5 Library Preparation and RNA-Seq

The library preparation methods (depending on the quantity and quality) and the sequencing platform (Ion Proton, Illumina and Roche) influence the profile and expressions levels of the transcripts (Selvaraju et al. 2017; Sendler et al. 2013; Mao et al. 2013). Hence, selection of appropriate method is highly challenging and crucial for sperm transcriptome profiling study. Ion Total RNA-seq kit v2 (Life technologies) and the SMARTer Ultra-Low Input RNA kit for the Illumina platform (Clonetech Laboratories, CA) were compared in our studies. Ion Proton library preparation method employs a ligase enhanced genome detection, which captures the fragmented RNA, whereas the ultra-low input library method increases the representation of GC-rich genes resulting in lower representation of rRNA. We also observed that the fragmentation and size selection steps during library preparation may not be required for biologically fragmented sperm RNA. Since sperm has intact as well as fragmented RNA, the use of random hexamers rather than poly-A based selection is effective to capture degraded RNAs. Before and after library preparation, presence of sperm RNA should be confirmed using primers for sperm-specific abundant transcript, for example, *PRM1*. In the case of sperm RNA-seq, it is necessary generate more reads (minimum 10× coverage) for meaningful interpretation of sequenced data.

As each sequencing platform has its own principle and technology, the selection of platforms depend on the objective and tissue/cell type (Liu et al. 2012). In addition, the read length and quality differ between the platforms, combining two different platforms, we obtained complete composition of biologically fragmented sperm total RNA from HF bull (Selvaraju et al. 2017). The tRNAs were observed to

be abundant in sperm transcriptome sequenced by the Ion Proton than Illumina platforms. Heterogeneity evidenced between the platforms (Ion Proton and Illumina) might be due to the library preparation and sequencing technology (Selvaraju et al. 2017).

7.6 Bioinformatics Analysis of Sperm RNA

The computational advancement in bioinformatics helps the biologist to understand and elucidate the functions of biomolecules. For RNA-seq data analysis, various bioinformatics tools are available as both freewares and paid software. Each tool uses a specific algorithm to predict transcripts with or without reference genome and calculate the transcripts abundances (RPKM/FPKM/TPM), expression level differences and other features such as splice junctions, SNPs, QTLs, motifs, retained elements, UTRs, coding sequences and promoter regions.

For an ideal RNA-seq experiment (any fresh/frozen tissue sample with good quantity and quality RNA), the bioinformatics pipeline is defined to some extent. However, for sperm samples, wherein the total RNA is highly fragmented, the established protocols are not available. Hence, for deciphering the biology behind the big data, the tools used at different stages of analysis need to be compared and an optimal bioinformatics pipeline to be established for the sperm RNA samples.

Analysis of bull sperm RNA-seq data using two different pipelines (CLC genomics workbench and Tophat-cufflinks-cuffdiff) revealed that though overall expression pattern and levels of the transcripts had a positive relationship, the ranking of transcripts based on abundance varied significantly. In addition, there is a need for establishment of bioinformatic pipeline for fragmented sperm RNA. A study from this lab revealed that TopHat2 was effective as compared to HISAT2 and STAR for mapping of the low expressed genes in sperm (Ramya et al. 2021). TopHat2 coupled with Cufflinks identified higher number of genes. edgeR and limma identified significantly more numbers of differentially expressed genes with biological relevance. This study established that TopHat2, Cufflinks and edgeR are suitable tools for the analysis of bovine sperm transcripts.

7.7 Transcript Composition and Quantification

The average yield of bovine sperm RNA ranges from 20 to 30 fg/spermatozoon. Buffalo sperm RNAs as compared with bovine are highly fragmented based on the bio-analyzer profile and RNA-seq data analysis. Transcriptome profiling reveals that majority of the sperm RNAs are protein coding in nature (Fig. 7.1). Sperm RNA comprised of ribosomal RNAs (rRNA), mitochondrial RNA, small non-coding RNAs and other RNAs (intronic retained elements, lncRNAs and Transcribed regions of Unknown Coding Potential (TUCP), short expressed regions, transposable elements and annotated non-coding RNAs-including snRNA, snoRNA and lncRNA) (Jodar et al. 2013; Sendler et al. 2013; Selvaraju et al. 2018). Both

mature and immature RNAs (with retained introns) were observed in the bovine sperm transcriptome (Fig. 7.2).

The total number of genes expressed in bull sperm ranges from 5000 to 7000. The sperm transcripts exists as both intact and fragmented. Their transcripts were distributed as only exonic reads, only intronic reads, also few reads covering the intergenic regions of the genome. *PRM1*, *CHMP5* and *YWHAZ* were observed to be the most abundant transcripts. The identified transcripts may significantly impact oocyte function, embryogenesis, trophectoderm development and pregnancy establishment (Selvaraju et al. 2017, 2021). In addition, the study also reveals that the genes governing sperm functional membrane integrity and acrosome integrity have a prospective effect on bull fertility (Selvaraju et al. 2021).

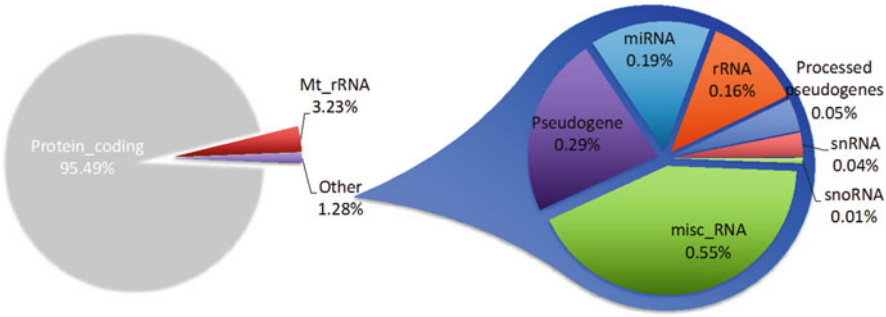

Fig. 7.1 Biotype composition of sperm transcripts (adapted from Selvaraju et al. 2018)

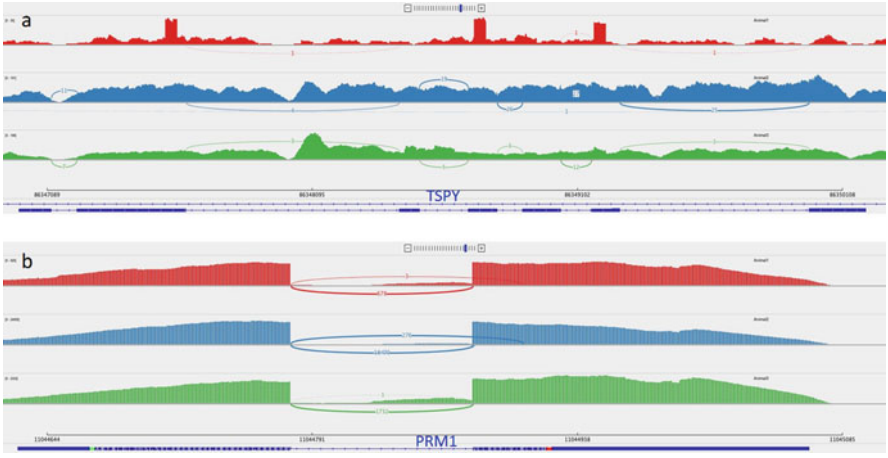

Fig. 7.2 The coverage and read distribution of *TSPY* suggests the presence of immature (**a**) and *PRM1* confirms the presence of mature and intact mRNA (**b**) in sperm (Selvaraju et al. 2017)

7.8 Functional Profiling of Sperm RNAs

The functional annotation of the spermatozoal RNAs is carried out using Uniprot KB, Database for Annotation, Visualization and Integrated Discovery (DAVID) and PANTHER databases. Enriched biological processes comprises of proteolysis, cell adhesion, ion channel regulation, metabolic activities, spermatogenesis, blastocyst development, organogenesis and anatomical structure development (Table 7.1). The molecular functions include peptidase activity, calcium ion binding, carbohydrate binding, nucleotide binding and oxidoreductase activity. The abundantly expressed transcripts were mainly involved in the axon guidance and oocyte meiosis pathways. Importantly, the transcripts associated with placental development are also abundantly expressed in the sperm (10 transcripts) indicating that the sperm-delivered transcripts may play an important role on the placental development. Sperm transcripts associated with the reproductive processes were involved in sperm-egg recognition, binding of sperm to zona pellucida and egg activation processes (Selvaraju et al. 2018). Overall, various studies on functional profiling of sperm transcripts from humans and animals strongly suggest that the expression pattern of the sperm transcripts can be used to understand the past events associated with spermatogenesis and future possible functional role on sperm function, fertilization and embryo development.

7.9 Sperm RNA Expression Pattern Can Be Used to Predict Bull Fertility Potential

Studies suggest that sperm transcripts differentially expressed between the bulls have relationship with fertility status. The transcripts present in the high fertile animals were involved in blastocyst development. The oxidoreductase activity and chemokine signalling pathway were upregulated in high fertile as compared to low fertile bulls. In low fertile animals, transcripts involved in purine metabolism were found to be unique. Studies from bovine revealed that spermatozoa transcripts expression levels can be used to assess the events associated with spermatogenic process (*BMP2* and *NGF*) and fertility (*UBE2D3, CASP3* and *HSFY2*). Additionally, the expression levels of apoptotic genes determine the fertility rate of bulls (Parthipan et al. 2017).

7.10 Sperm RNA Retained Regions and Predicting Fertility

The sperm RNA elements can be a potential tool to predict fertility status of the bull. In human study, 40% of the exonically retained elements are associated with sperm fertilisation process and 20% in cellular processes; however, the functions of remaining 40% of them are not known (Jodar et al. 2015). Sperm-retained RNA elements were observed to be abundant in bull (Selvaraju et al. 2017). The intronic retained regions were relatively high when compared to exonic retained regions in

Table 7.1 The spermatozoal transcripts associated with the key reproductive process (extracts from Selvaraju et al. 2018)

Biological process	Transcripts	Molecular functions
Spermatogenesis	*PRM1, CHMP5, WHAZ, DNAJC2, TNP1*	Germ cell development Regulation of cell division Chromatin modification
Sperm maturation	*PPARGC1A, FLCN, ADPGK, ATP5F1, PPARGC1A*	Microtubule cytoskeleton organization Regulation of energy homeostasis
Sperm function	*CATSPER3, PLCB1, FYN, NCK1, NCK2*	Regulation of cell motility Membrane integrity Positive regulation of acrosome reaction
Fertilization	*PRSS37, HSPA1L, CCT2, ZP2, ZP3*	Sperm-egg recognition Binding of sperm to zona pellucida Sperm capacitation
Oocyte genome activation	*PRSS37, HSPA1L, PLCZ1, WBP2NL, ZP2*	Egg activation Oocyte genome activation Regulation of phosphorylation
Embryogenesis	*BCL2L11, BRCA1, HNF1B, CDH1, CDX2*	Embryonic development Embryonic organ morphogenesis Embryonic development ending in birth
Placental development	*HNF1A, NANOG, SMAD1, ARNT, CDX2*	Embryonic placenta development Ectoderm, mesoderm and endoderm development

bull spermatozoa (Fig. 7.3a). The other retained elements also observed in intergenic regions (intron-exon), as well as in 5′ and 3′ UTRs (Fig. 7.3b). The translation of sperm mRNA by maternal ribosomes is still unclear but could be hypothesized that the sperm RNA retained regions may bind to the complementary/selected maternal RNAs and thus regulate the early embryogenesis. These elements can be used as markers for selection of bulls for the breeding program.

7.11 Splicing Mechanisms in Sperm

Alternative splicing (AS) occurring at the transcriptional, post-transcriptional and epigenetic levels regulates the developmental process of mature spermatozoa. AS also results in the production of different proteins from the same gene. The aberrant transcripts were associated with the reduced reproductive performance (Guo et al. 2013). The whole transcriptome sequencing of sperm has the benefit of studying the AS mechanisms as well as their functional associations in the fertilization process. For example, *SPEF2* splice variants are differentially expressed in the bovine testis,

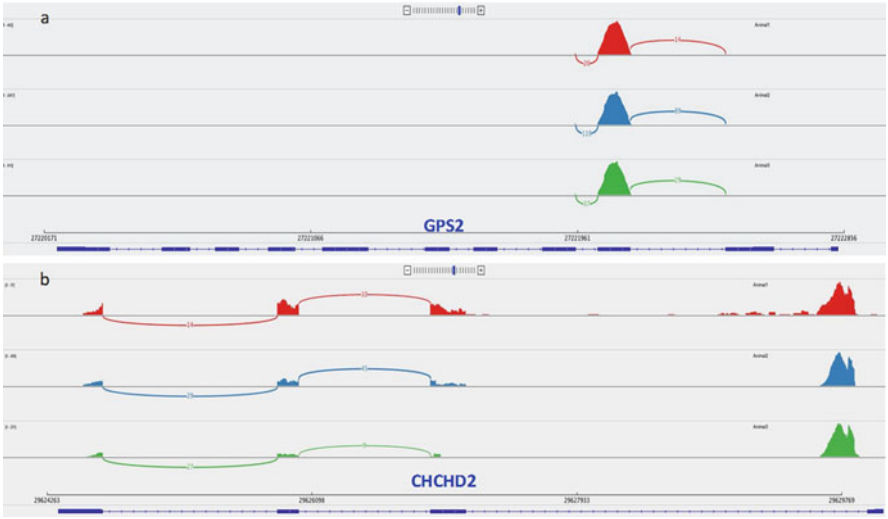

Fig. 7.3 Retained regions in bull sperm transcripts. As compared to the exonic retained regions (**a**), intronic retained regions (**b**) were relatively high in spermatozoa (Selvaraju et al. 2017)

epididymis and sperm. These are involved in sperm motility (Guo et al. 2013). The gene *PMCA4* has two splice variants and the knock out studies showed that these variants are essential for hyperactivated sperm motility during fertilization (Okunade et al. 2004). Similarly, alternative splicing of *IZUMO1* facilitates sperm-oocyte fusion in mice (Saito et al. 2019).

7.12 Non-coding RNAs in Sperm

Non-coding RNAs play a crucial role in post-transcriptional gene regulations during spermatogenesis and oogenesis. Non-coding RNAs such as microRNAs (miRNAs), piwi interacting RNAs (piRNAs) and long non-coding RNAs (lncRNAs) have their roles in the gametogenesis and sperm motility (reviewed in Selvaraju et al. 2018; Wang et al. 2019).

7.13 Sperm microRNA

The roles of spermatozoal small non-coding RNAs (sncRNAs) are intensely pursued to understand the spermatogenesis events as well as paternal contribution on fertilization, embryo development and likely influence on the phenotype of the offspring (Krawetz et al. 2011; Ni et al. 2011). The sncRNA might regulate post-transcriptional gene expression. The miRNAs such as miR34c-5p, miR124 are observed to be abundant in human spermatozoa. These miRNAs might regulate sperm function oocyte maturation and different stages of embryo development. For

example, miR34c-5p is involved in the cleavage of the embryo (Liu et al. 2012). Studies from Krawetz lab also suggest that the expression of miR34c-5p may vary between individuals and the expression level may have relationship with pregnancy outcome (Selvaraju et al. 2013).

7.14 Sperm Long Non-coding RNA

lncRNAs are involved in transcriptional regulation of spermatocyte-specific gene expression. LncRNA-Tcam1 and lncRNA-HSVIII were identified to be crucial for pachytene spermatocytes. NLC1-C has been associated with male infertility through the control of miRNA expression via RNA-binding proteins in human spermatogenesis (Luk et al. 2015)

7.15 Sperm Reverse Transcriptase Activity

Though studies indicate that sperm are transcriptionally silent, they can actively uptake, transport and integrate exogenous molecules into it. RNA sequencing of sperm strongly indicates the presence of long interspersed nucleotide elements (LINE-1). The LINE-1 encoded reverse transcriptase activity, reverse transcribe RNA into cDNA for delivering it to the oocyte and embryo (Spadafora 2008; Simoes et al. 2013). Such exogenous genes are reported to be expressed in the subsequent generations (Sciamanna et al. 2003; Zhang et al. 2012) and may influence the phenotype of the offspring. It is suggested that such endogenous reverse transcriptase activity of the sperm can generate transcriptionally competent and non-integrated retrogenes that are transmitted to the progeny. Such mechanisms can have implications in the production of transgenic animals, management of stress and health apart from evolutionary processes (Sciamanna et al. 2003; Sciamanna and Spadafora 2012; Bosch et al. 2015). The sperm-delivered sequences may remain as extra-chromosomal and transmitted to the next generation in a non-Mendelian fashion (Sciamanna et al. 2009), which is similar to RNA-mediated paramutation inheritances (Rassoulzadegan et al. 2006; Rando 2012). Another feature of sperm that support sperm mediated gene transfer is the presence of endogenous reverse transcriptase activity (Giordano et al. 2000).

7.16 Future Directions

Sperm RNA profiling may play an important role in selecting high fertile bulls for breeding. In order to make sperm RNA profiling very effective in fertility diagnostic field, many steps from RNA isolation to interpretation of big data need to be standardised and validated in large numbers of animals.

7.17 Conclusion

The whole transcriptome profiling of sperm RNAs by next-generation sequencing techniques has been established. The total RNA isolation methods are standardized for each species, and the sperm RNA composition and abundance vary between the animals differing in fertility. The library preparation methods and sequencing platforms influence the sperm RNA profile and expression levels. Sperm expressed genes are associated with spermatogenesis, sperm function, fertilization and early embryonic development. The sperm RNA profiling might be a non-invasive approach to predict and improve fertility in livestock.

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Semen Proteomics and Metabolomics: Emerging Tools for Discovering Fertility Markers

8

A. Kumaresan, M. K. Muhammad Aslam, Mohua Das Gupta, and Manish Kumar Sinha

Abstract

In dairy bovines, semen samples from an individual bull are used for artificial insemination in many cows to explore the full potential of bull genetics for maximizing production. Therefore, proper determination of the fertility of bulls, before employing them for breeding, is of utmost importance for minimizing the wastage of valuable resources. However, existing data indicate that the pregnancy rates among breeding bulls, selected based on breeding soundness evaluation, differed by 20–25%. Recently, it is well understood that besides the sperm phenotypic characteristics and functionalities, the molecular health of sperm plays a vital role in fertilization and embryonic development. This opens great scope for development of additional evaluation methods that can determine the intrinsic fertility of bulls with greater accuracy. In this context, the use of the novel "omics" approach, specifically genomics, proteomics, transcriptomics, and metabolomics tools, has emerged as a bright spot and open great scope for identifying semen molecules, which can predict the latent fertility of semen samples and the reproductive efficiency of bulls with great accuracy. Among these technologies, the proteomics and metabolomics approaches have been widely accepted as the primary choice for identifying the fertility biomarkers in the sperm and seminal plasma of humans and animals. This chapter describes the

A. Kumaresan (✉) · M. K. Sinha
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India
e-mail: A.Kumaresan@icar.gov.in

M. K. Muhammad Aslam
Base Farm, Kerala Veterinary and Animal Sciences University, Idukki, Kerala, India

M. D. Gupta
College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

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methods, status, and scope for discovering fertility biomarkers in bull semen using the proteomics and metabolomics approaches that can predict the fertility more precisely.

Keywords

Breeding bulls · Sperm · Seminal plasma · Proteomics · Metabolomics · Fertility

8.1 Introduction

Intrinsically, both female and male contribute to infertility but the problems in female have drawn attention and improvised through assisted reproductive technologies. Nevertheless, the infertility in bull was overlooked over a period of long time, and the acceptable quality semen producing ability decreased through generation and highlighted a significant change in the aura of dairy industry in terms of declining conception rate (Roberts and Jarvi 2009). Basically, the male infertility is deep seated in different level of omics such as genomic (Taylor et al. 2018), transcriptomic (Tripathi et al. 2014), proteomic (Aslam et al. 2015), metabolomic (Saraf et al. 2020; DasGupta et al. 2021a), lipidomic, and glycemic levels. Despite the fact, the diagnostic techniques of male infertility hanged on outdated semen assessment protocol, which remained unchanged from the previous years. Additionally, poor quality ejaculate also came into light as a major concern for colossal loss in dairy industry. Thusly, the rising incidence of poor seminal traits and declining status of fertility of an individual has diverted the area of research into the world of "omics" wherein proteomics and metabolomics approaches appeared as complementary to the existing omics technology and venture towards the identification of specific biomarkers and altered pathways in male infertility from body fluids/cells/tissue of an individual.

In recent past, the first step of proteomics and metabolomics has been applied at sperm, seminal plasma (Peddinti et al. 2008; D'Amours et al. 2010; Aslam et al. 2015; Saraf et al. 2020; DasGupta et al. 2021a), and testicular level (Tripathi et al. 2014; Tomar et al. 2021) to elucidate the etiology of declining trends of semen quality and male fertility. Fundamental importance of a wide variety of proteins such as FAA/DNase I like protein, BSP, osteopontin, enolase, MDH, PG- D-Synthetase, Phospholipase A2, spermadhesins, clusterin, ubiquitin, aSFP, and metabolites such as fructose, sucrose, phospholipid, taurine, calcium, potassium, cholesterol, intracellular antioxidant enzymes and minerals in sperm and glutamate, pyruvate, oxaloacetate, lactate, taurine in seminal plasma of varied species have been explored to enlighten the prevailing concept of male infertility (Asadpour 2012; Duan and Goldberg 2003), and portrayed its role in various metabolic processes, sperm motility, viability, and fertility of spermatozoa through omics technology. Likewise, meta-analysis of human seminal traits showed a rapid fall in fructose and alpha-glucosidase with increasing age of individuals (Molina et al. 2010) and reported that fluctuations in concentration/level of above-mentioned metabolites have instigated

alteration in male fertility level (Tvrdá et al. 2019). Ideally, seminal metabolites have positive correlation with the overall health of an individual and evaluating at an early age provides an insight on fertility potential in human (Drabovich et al. 2014) as well in domestic livestock species (Kňažická et al. 2020; Intasqui et al. 2016; Argov-Argaman et al. 2013). Although the attributed etiology of such fluctuations was documented as pathology of reproductive tract, genetic disorders, and poor management, but the exact cause is still not clear, there are plenty of areas to explore the hidden mystery of increasing male infertility. Moreover, the knowledge about the identified proteome and metabolome of sperm and seminal plasma is still in its infant stage and so far, very few studies have been executed in domestic livestock species. Therefore, the central to this chapter is to compile the identified sperm and seminal plasma proteins and metabolites, and to delineate their role in male fertility besides discussing the concept of emerging applications of proteomics and metabolomics in bovine male fertility.

8.2 Semen Proteomics and Male Fertility

Proteomics can be defined as the systematic analysis of all the proteins in a tissue or cell and aims to identify the expression levels of all proteins of one functional state of a biological system (Carrell et al. 2016). It advocates the qualitative and quantitative comparison of proteomes to identify the cellular mechanisms associated with various biological systems. Proteomics also includes studying the structure, function, and interactions of the expressed complement of proteins (Kwon et al. 2015). It is a mass screening approach, where the overall distribution of proteins in the given system is documented, and the interesting ones are identified and characterized ultimately to elucidate their functional roles and relationships (Cahill 2001). As proteins hold key responsibility in carrying out various cellular functions, it is crucial to have detailed knowledge about the quantitative and qualitative expression of proteins in cells and tissues comprehensively for a thorough understanding of these processes.

Although genomics has achieved new heights and is extensively being used in various research applications, certain limitations make it less promising for semen evaluation. Mature spermatozoa are transcriptionally inactive and the number of transcripts in spermatozoa is low (Kierszenbaum and Tres 1975; Gilbert et al. 2007). Moreover, studying the gene expression alone does not provide actual ideas about the fertility factors, as several other factors regulate the expression, which may also differ between the cells. In this context, since the sperm cells are terminally differentiated cells and lack the ability to synthesize new proteins, the proteomic analysis may help to determine their accurate fertilizing ability. Hence in the post-genomic period, the proteomic approach gained prominence in andrology for discovering the fertility biomarkers and identifying the underlying mechanism of infertility or sperm dysfunction (Wright et al. 2012).

The spermatozoa interact with several proteins during various phases of their lifecycle, starting from spermatogenesis, sperm maturation, capacitation, etc., to achieve the complete fertilization potential. Any defects or altered expression of

proteins at any of these stages may lead to fertilization failure. The proteomic approach helped to find out answers for many of hitherto unanswered questions related to sperm function and male fertility and as a result, the discovery of fertility biomarkers in sperm and seminal plasma has gained significant attention during the past few years (Peddinti et al. 2008; Soggiu et al. 2013; Aslam et al. 2014, 2018, 2019).

8.3 Techniques for Semen Proteomic Studies

Proteomics technique employs a mass screening approach, where the expression of all the proteins in the biological systems is studied. Generally, two different approaches are being used for the proteomic studies of semen samples, the conventional gel-based approach and the recent shotgun or bottom-up approach.

8.3.1 Gel-Based Method

Two-dimensional (2D) gel electrophoresis coupled with mass spectrometry (MS) technique was conventionally being used to identify the proteomic biomarkers of fertility in the seminal plasma and spermatozoa of bulls.

In the gel-based approach, the proteins in the given sample (either seminal plasma, spermatozoa, or testicular cells) were separated using 2D electrophoresis, based on their iso-electric focusing points and molecular weights. For identifying the fertility-related proteins, comparing the gels of different samples from bulls with varying fertility index is required. The 2D gels are analyzed using computer software for finding differentially expressed spots, which are then excised, enzymatically digested, and identified using mass spectrometric methods such as MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) technique. The advances in the 2D gel-based method have led to many breakthrough studies in semen and other biological fluids to unravel the proteome and identification of biomarkers.

DIGE (Difference Gel Electrophoresis) is a modified version of 2D-gel electrophoresis, which can determine the differentially expressed protein spots with more accuracy. In DIGE, two different sets of samples can be analyzed in a single run, with the minimum requirements for time and resources (Baker et al. 2010; Oliva et al. 2008). It has superiority over other gel-based techniques regarding high sensitivity and linearity of the dyes utilized, minimum inter-gel variability, and reduced experimental bias. Moreover, along with the control and experimental samples, a pooled internal standard is also loaded, which considerably improves the quantification accuracy and statistical confidence (Alban et al. 2003).

The gel-based techniques have certain intrinsic disadvantages in terms of sensitivity and the power to discover a greater number of proteins and scarce proteins in the samples. There might be the comigration of multiple proteins into same spots which makes the results erroneous. In addition, there are limitations in adopting automation technologies in gel-based procedures (Abdallah et al. 2012).

8.3.2 High-Throughput Shotgun Method

The advancement and popularity of mass spectrometric methods helped to analyze maximum number of proteins in samples with more accuracy. The shotgun approach using LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) method has been widely used to identify the differentially expressed proteins in experimental samples. The LC-MS/MS approach combines the solute separation ability of HPLC with the detection capacity of a mass spectrometer. HPLC can separate proteins or peptides based on some unique properties such as charge, size, hydrophobicity, and the presence of specific tags or amino acids. As HPLC is coupled to an MS/MS (tandem mass spectrometer) with an interface, rapid separation of the complex protein mixture and identification of its components are possible with accuracy (Baker et al. 2010).

For the comparative analysis of protein expression, various techniques which employ the labelling of peptides such as tandem mass tags (TMTs), isobaric tag for relative and absolute quantitation (iTRAQ) labelling, stable isotope labelling by amino acids in cell culture (SILAC) and isotope-coded affinity tag (ICAT) are generally used. Among these techniques, iTRAQ labelling has been widely used in proteomics studies of spermatozoa (Liu et al. 2018; Agarwal et al. 2020). For the last few years, most of the researchers trusted the throughput shotgun approach, using LC-MS/MS technique for comparative proteomics and identification of proteomic biomarkers of fertility in the spermatozoa and seminal plasma of animals (Somashekar et al. 2017; Viana et al. 2018).

8.4 Approaches in Fertility Biomarker Discoveries

The widely followed approach for identification of fertility biomarkers is the use of comparative proteomics, in which the sperm, seminal plasma, or testicular cells of bulls with varying fertility index were compared to identify the differentially expressed proteins. The differential expression is quantified, and the most relevant and reliable proteins are selected based on certain procedures for further verification and validation. The proteomic approach for identification of fertility biomarkers in semen is given in Fig. 8.1.

8.4.1 Selection of Bulls for the Studies

The fertility score or conception rates (NRR/CR) of individual bulls can be determined based on the number of insemination and pregnancy outcomes per bull. Various factors that influence the fertility performance of sires such as breeding events, environmental and herd management practices, etc. are adjusted using appropriate statistical models to minimize the error. The least squares technique for non-orthogonal data can be used to estimate the effect of non-genetic factors (Singh et al. 2017). The high- and low-fertile bulls can be selected, based on the

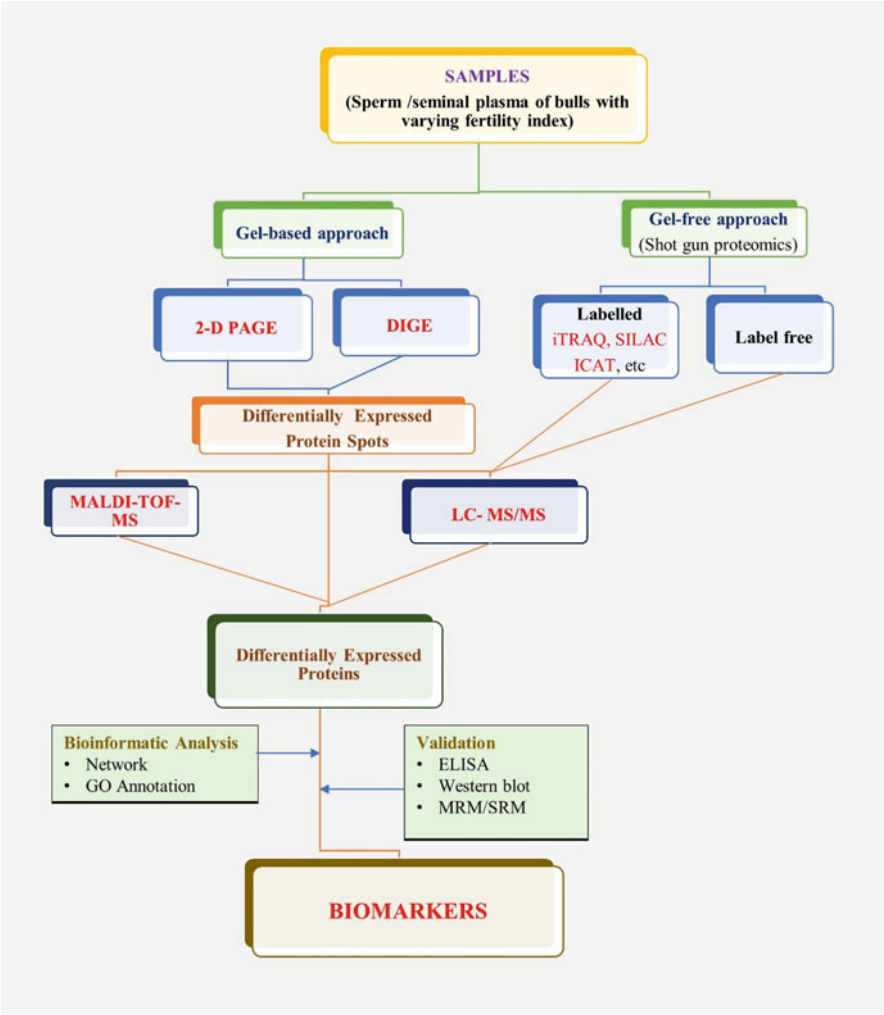

Fig. 8.1 Approaches and techniques generally used for discovering proteomic biomarkers

difference from standard deviation. Generally, bulls with CR >1 + SD may be considered as high fertile and those with CR <1-SD as low-fertile bulls (Saraf et al. 2017; Viana et al. 2018; Aslam et al. 2019). It is preferred to have minimum six bulls in each group, but there are practical difficulties in obtaining the fertility data of several bulls from the field, and studies with two or three bulls per group were also performed previously (Aslam et al. 2014; Peddinti et al. 2008).

8.4.2 Experimental Procedure and Methods

The semen samples can be collected using standard procedures followed in bull stations, and immediately after collection, suitable protease inhibitor cocktail should be added to the semen for preventing the action of proteases. The cellular part and seminal plasma can be separated as required using different centrifugation protocols. If the seminal plasma is preferred for analysis, it can be obtained through direct centrifugation of semen samples firstly at 700 × *g* for 10 min, followed by another centrifugation of the supernatant at 10,000 × *g* for 60 min, both at 4 °C (Viana et al. 2018). The cellular part contains spermatozoa, immature germ cells, and somatic cells such as leucocytes and epithelial cells. The common purification method adopted to isolate the spermatozoa is centrifugation with colloidal silica density gradients, and the preferred one is Percoll based centrifugation. The semen sample is layered on the top of a Percoll gradient and centrifuged at 800 × *g* for 20 min at 4 °C to recover the purified spermatozoa from the pellet (de Mateo et al. 2013). For isolation of motile spermatozoa, the swim up technique is also employed (Henkel and Schill 2003). After isolation of the desired part, the samples can be stored at −80 °C till further analysis. The sperm cells can be disrupted through sonication or using detergents (e.g., Triton X-100) to extract the intracellular proteins for proteomic analysis.

The next steps in the procedure are the isolation and identification of the proteins, especially the differentially expressed proteins. Previously, 2D gel electrophoresis or DIGE coupled with mass spectrometry technique was used for extraction and identification, but the recent experiments make use of liquid chromatography combined with mass spectrometric techniques like LC-MS/MS. The detailed procedure of these techniques can be accessed from previous reports (Aslam et al. 2014; Somashekar et al. 2017). After the identification of proteins, if the number of proteins is very high, it is convenient to categorize them using GO annotation software, based on the biological process, molecular function, and cellular component for understanding the mechanisms of action. The results of GO annotation can be expressed in tabular format or represented graphically. There is many software available for the purpose, and the popular ones are PANTHER, STRAP, Blast2Go, etc. The functional interaction between the differentially expressed proteins in each experiment can be analyzed using STRING v. 9.1 (http://string-db.org) database as described by Szklarczyk et al. (2010). STRING is a meta-resource that aggregates most of the available information on protein–protein associations, scores, and weights, and augments it with predicted interactions, as well as results of automatic literature-mining searches.

Once the differentially expressed proteins are identified, based on various criteria, the proteins of interest can be selected for further validation in a large population. Previously, techniques like western blotting and ELISA were used for validation of identified biomarker molecules but recently targeted MS-based strategies like MRM (Multiple Reaction Monitoring) have been established as a more accurate alternative. The MS-based techniques permit specific simultaneous quantification of several biomarkers with accuracy and reliability.

8.5 Major Proteomic Biomarkers Identified in Sperm and Seminal Plasma

Proteins in the semen may be present either in seminal plasma or in the spermatozoa. Some seminal plasma proteins get attached to the sperm surface and modulates different sperm functions. Seminal plasma is a complex mixture of proteins, salt, sugar, citric acid, prostaglandins, electrolytes, etc. (Mann 1964). Though there are interspecies differences in the number and concentrations of proteins based on the nature of accessory sex glands present, proteins are the key component of seminal plasma in the case of bovines. Most proteins present in the seminal plasma are originated from the blood, or synthesized by the testes, epididymis, and accessory sex glands (Kato et al. 1985; Manjunath et al. 1994). These proteins are involved in the acquisition of motility of spermatozoa (Acott et al. 1983), ionic equilibration, protective action (Markandeya and Pargaonkar 1990), capacitation of spermatozoa (Stewart-Savage 1993), sperm–zona interaction, and the fertilizing capacity. The proteins identified to be associated with fertility are detailed in Table 8.1. Some of the important bull fertility proteins reported by researchers are discussed below.

8.5.1 Bovine Seminal Plasma (BSP) Proteins

A major part of the total protein content of the bovine seminal plasma is constituted by the bovine seminal plasma (BSP) proteins, which are heparin-binding proteins secreted by seminal vesicles (Moura et al. 2007). BSP family consists of four proteins, namely BSP-A1, BSP-A2, BSP-A3, and BSP- 30 kDa, of which BSP-A1 and BSP-A2 together were previously known as PDC-109 (protein with N-terminus aspartic acid, D, and carboxy-terminus cystine, having 109 amino acids; Esch et al. 1983), and contributed a major part of BSP proteins (Scheit et al. 1988). During ejaculation, millions of BSP protein molecules attach to each sperm (Calvete et al. 1995) by binding to the choline phospholipids such as phosphatidylcholine (PC) and sphingomyelin present on the outer leaflet of the sperm plasma membrane (Sankhala and Swamy 2010). These proteins induce cholesterol and phospholipids efflux from sperm in a dose and time-dependent manner (Thérien et al. 1998) leading to destabilization of the membrane. The multifunctional properties of BSP A1/A2 include regulation of sperm motility (Sánchez-Luengo et al. 2004), oviductal sperm reservoir formation (Gwathmey et al. 2003), modulation of sperm capacitation, and acrosome reaction (Srivastava et al. 2013).

Even though this protein is essential for capacitation and hyperactivation, in the case of cryopreservation and long storage, it has many deleterious effects which affect the cryo-survivability of the spermatozoa (Bergeron and Manjunath 2006; Srivastava et al. 2013). Hence, it can be believed that the overexpression of BSP proteins in ejaculated spermatozoa leads to a reduction in its fertilizing potential (Aslam et al. 2018).

Table 8.1 Proteomic biomarkers in seminal plasma and spermatozoa of bulls and their association with fertility

Marker	Association	Function	Reference
Seminal plasma			
FAA/DNase I like protein	Direct	Capacitation	Bellin et al. (1998), McCauley et al. (1999), Moura et al. (2006, 2007)
BSP	Direct	Capacitation	Sánchez-Luengo et al. (2004), Moura et al. (2006)
Osteopontin	Direct	Egg binding, fertilization	Killian et al. (1993), Cancel et al. (1997), Moura et al. (2006)
PG-D-Synthetase	Direct	Development and maturation of sperm	Killian et al. (1993), Gerena et al. (1998)
Phospholipase A2	Direct	Maturation of sperm, acrosome reaction, fertilization	Bao et al. (2004), Moura et al. (2006)
Spermadhesin Z13	Inverse	Adversely affects motility	Killian et al. (1993), Moura et al. (2006)
aSFP	Direct	Sperm metabolism, prevents oxidative damage	Schöneck et al. (1996), Jobim et al. (2004)
Nucleobindin	Direct	Protect sperm and prevent premature acrosome reaction	Moura et al. (2007, 2010)
Clusterin	Inverse	Sperm damage	Ibrahim et al. (1999), Moura et al. (2006)
Ubiquitin	Inverse	Defective sperm	Frenette et al. (2002)
IGF I	Direct	Motility	Henricks et al. (1998)
Spermatozoa			
PH 20	Direct	Zona binding	Lalancette et al. (2001)
P25b	Direct	Acrosome reaction	Parent et al. (1999)
COX3	Direct	Oxidative respiration	Peddinti et al. (2008)
ATP5B/ ATP5D	Direct	Energy synthesis	Peddinti et al. (2008), Aslam et al. (2018)
CKII	Direct	Sperm chromatin decondensation after fusion	Peddinti et al. (2008)
AKAP4	Direct	Sperm motility	Peddinti et al. (2008)
ODF2	Direct	Sperm structure integrity	Wang (2010)
MnSOD	Inverse	Protect sperm from oxidation	Wang (2010)
SP32	Direct	Capacitation	Wang (2010)
PEPB1	Direct	Decapacitation factor	D'Amours et al. (2010)
AK1	Direct	Hyperactivated motility	D'Amours et al. (2010)
PSMA6	Inverse	Protease activity	D'Amours et al. (2010)
Tektin 4	Direct	Sperm motility	Thepparat et al. (2012)
ENO1	Direct	Energy metabolism, Sperm motility	Park et al. (2012), Soggiu et al. (2013), Aslam et al. (2018)
MDH2	Direct	Energy metabolism	Aslam et al. (2018)
PDC-109	Inverse	Sperm capacitation	Aslam et al. (2018)

8.5.2 Alpha Enolase

Alpha Enolase (ENO1) is a multifunctional enzyme that catalyzes the conversion of 2- phosphoglycerate to phosphoenol pyruvate in glycolysis. They play an important role in cellular motility by preserving energy metabolism pathways. In the case of spermatozoa, ENO1 is mostly present in the tail part and regulates the uninterrupted supply of energy for the motility and also helps to protect male gametes against oxidative stress (Gitlits et al. 2000). Many studies proved that Alpha Enolase is a promising biomarker of bull fertility (Aslam et al. 2018).

8.5.3 ATP5D

ATP synthase is a key enzyme in bioenergetics of a living cell that provides energy for the cell to use through the synthesis of adenosine triphosphate (ATP). It consists of five subunits and catalyzes the production of ATP from ADP in the presence of a proton gradient across the mitochondrial membrane, and this ATP is utilized for the motility of sperm and capacitation (Peddinti et al. 2008). High homology is reported between different subunits of F1 ATPase, and ATP5A, B, and D are already reported as a fertility-related protein in bulls (Peddinti et al. 2008; Park et al. 2012; Soggiu et al. 2013; Aslam et al. 2018).

8.5.4 MDH2

MDH2 is a TCA cycle enzyme that catalyzes the oxidation of malate into oxaloacetate using NAD+/NADH as a cofactor, in the last step of the Krebs cycle (Sharma et al. 2013). So, the reduction in MDH2 may lead to a reduction in the internal energy distribution of spermatozoa affecting its motility, capacitation, hyperactivation, and fertilizing ability (Soggiu et al. 2013). The expression of MDH2 was very much related to fertility and reported as biomarkers of fertility in humans (Sharma et al. 2013), bulls (Soggiu et al. 2013; Aslam et al. 2018), and stallions (Novak et al. 2010).

8.5.5 Osteopontin (OPN)

Osteopontin, a multifunctional extracellular matrix protein found in many tissues and body fluids is reported to have closely associated with male fertility. Killian et al. (1993) identified four fertility associated proteins in the seminal plasma of Holstein Friesian bulls using 2D PAGE technique. They reported that two proteins (26 kDa, pI 6.2; 55 kDa, pI 4.5) occurred with greater frequency and density in bulls of higher fertility and two proteins (16 kDa, pI 4.1; 16 kDa, pI 6.7) were more prominent in bulls of lower fertility. These proteins, overexpressed in high-fertile bulls, were later

identified as osteopontin (55 kDa; Cancel et al. 1997) and lipocalin-type prostaglandin D synthase (26 kDa; Gerena et al. 1998).

It is reported that OPN was present at multiple molecular weight forms in the reproductive tract of bulls and plays an important role in the fertilization through its interaction with the integrin and other proteins of the oocyte plasma membrane (Erikson et al. 2007).

8.5.6 Thimet Oligopeptidase-1 (TOP1)

TOP is an enzyme which catalyzes the hydrolysis of gonadotropin-releasing hormone (GnRH) in vitro and present in high quantities in testes (Orlowski et al. 1989). Even though the sperm functions of these enzyme are not sufficiently reported, it was apparently present in high concentrations of the enzyme in cytoplasm of spermatids (Pineau et al. 1999). In our study, we observed that TOP1 is over expressed in the spermatozoa of high-fertile crossbred bulls compared to their low-fertile counterparts (Aslam et al. 2018).

The immunolocalization of some important fertility-related proteins on the spermatozoa of bulls using FITC tagged antibodies is depicted in Fig. 8.2.

8.6 Semen Metabolomics and Male Fertility

Towards the end of 1960s, the pioneering work of Horning led the foundation of a novel techniques termed metabolomics. The comprehensive profiling of urine metabolites by using gas chromatography opened an area to venture into a new concept of biological fingerprint. Various techniques including NMR spectroscopy, Raman spectroscopy, gas chromatography/liquid chromatography-mass spectrometry, HPLC-MS and capillary electrophoresis MS have been applied to identify metabolites in biological cell lines, tissues, and body fluid. However, nuclear magnetic resonance (NMR) appears as one of the most widely used techniques for identification of metabolites. Kumar et al. (2015) executed NMR method as a confirmation of principle study to identify fertility associated metabolites in blood serum and seminal plasma of dairy bull. This method identified citrate (2.50 ppm), leucine (0.78 ppm), isoleucine (0.74 ppm), taurine/tryptamine (3.34–3.38 ppm) peaks in seminal plasma and citrulline (1.54 ppm), glycogen (3.98), asparagine (2.90–2.94 ppm) in serum and suggested use of metabolites as biomarkers in breeding bull. Meanwhile, the never-ending progress in the area of omics introduced MS-based methods with some advantages over NMR in terms of sensitivity (Courant et al. 2013). Since the metabolome found to regulate various metabolic process and sperm functions, several articles on MS-based metabolomics have been published in clinical diagnostics, therapeutic interventions, normal biological process, pathogenic process, and candidate biomarkers (Saraf et al. 2020; Korosi et al. 2017; Lucio et al. 2017). Identification of phospholipid as sperm motility markers in canine (Korosi et al. 2017) by MS-based metabolomic approach has also thrown

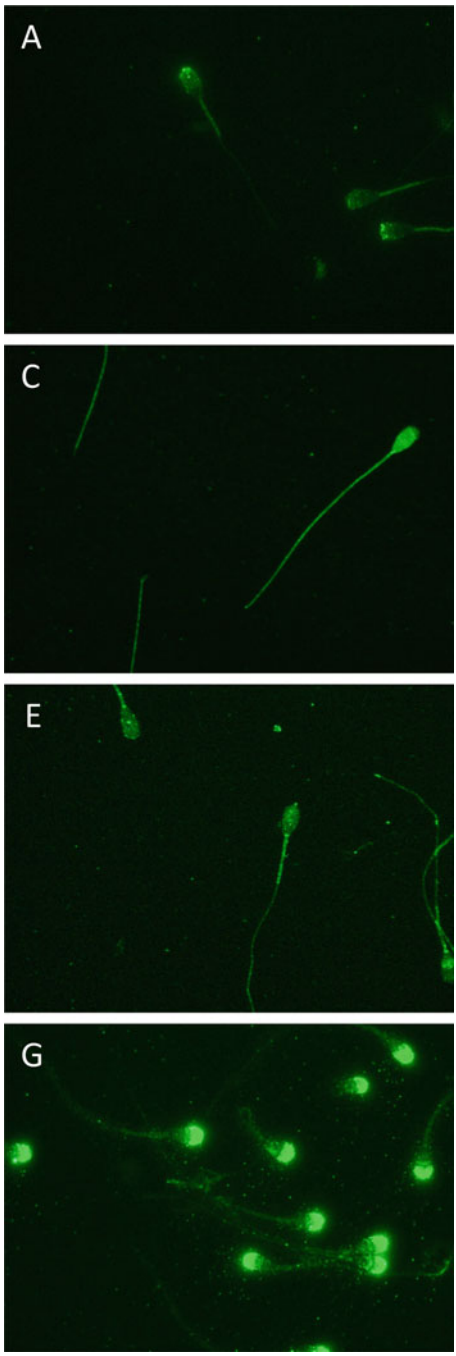

Fig. 8.2 Immunolocalization of selected sperm proteins in spermatozoa of crossbred bull using FITC tagged secondary antibodies. [A] Alpha Enolase, [C] Mitochondrial malate dehydrogenase-2, [E] F_1 ATP synthase subunit D, and [G] PDC-109

light on the existing alteration in semen quality and noted the urgent need of in-depth analysis of smallest molecule to better correlate with the male infertility.

Classification of Metabolites: Generally, metabolites (tiny molecule) being the end product of any biological pathways, weighing <150 kd, reflecting a downstream events of gene expression and considered as more closer to the actual phenotype (Patti et al. 2012; Kovac et al. 2013), appear as superior and reliable representative of phenotypic traits, as compared to proteomics or genomics, whose results are not complicated by post-transcriptional and post-translational processes (ter Kuile and Westerhoff 2001). Therefore, within the omics technology, perhaps metabolomics stands closer to disease as well as environmental effect on reproduction. Briefly, extraction of metabolites is carried with a multi-solvent method, using methanol: acetonitrile: water in the ratios of 2:2:1 (Liebeke and Bundy 2012), and the extracted metabolites are separated in an untargeted fashion by liquid chromatography method which enables detection of thousands of metabolites from a single analysis (Fig. 8.3). But, structural annotation of all the identified metabolites remains a challenge because of limited information available on reference spectra in the online libraries and databases. These libraries are created with the commercially available structural database and compounds. Since the libraries and database are constantly growing, a large number of biomolecular structures are not available and thus little structural insight can be obtained from the identified fragments in metabolomics experiment. Besides, it is also not possible to delineate a comprehensive structural picture of identified metabolites. Identified endogenous metabolites are categorized into lipid, phospholipid, glycerophospholipid, amines, amino acids, fats, and common fatty acid based on functional enrichment of chemical compound in MBrole 2.0 (http://csbg.cnb.csic.es/mbrole2) and divided in to two types, primary and secondary. However, primary metabolites have a direct role in several metabolic process and sperm functions, whereas secondary metabolites have an indirect role and produced during the break down of primary metabolites. Secondary metabolites include steroids, phenolics, alkaloids, resins, tannins, lignins, and essential oils. Some identified metabolites are classified into super classes on the basis of HMDB and PubChem website information (Table 8.2).

8.7 Interaction of Metabolites Between Sperm and Seminal Plasma

The low molecular compound present in sperm and seminal plasma can regulate the complex cascade of fertilization, varied with respect to fertility status of an individual/ bull and can also act as a fertility marker. Generally, citrate acts as a chelator of calcium ions and limits sperm capacitation and spontaneous acrosome reaction (Ford and Harrison 1984), whereas taurine found to enhance post-thaw motility and survivality of sperm (Chillar et al. 2012). Isoleucine and leucine alter calcium transport across plasma membrane and hence alters motility, capacitation, and acrosome reaction (Rufo et al. 1982), whereas tryptamine promotes the acrosomal reaction and regulates sperm motility in capacitated hamster sperm (Jimnez-Trejo

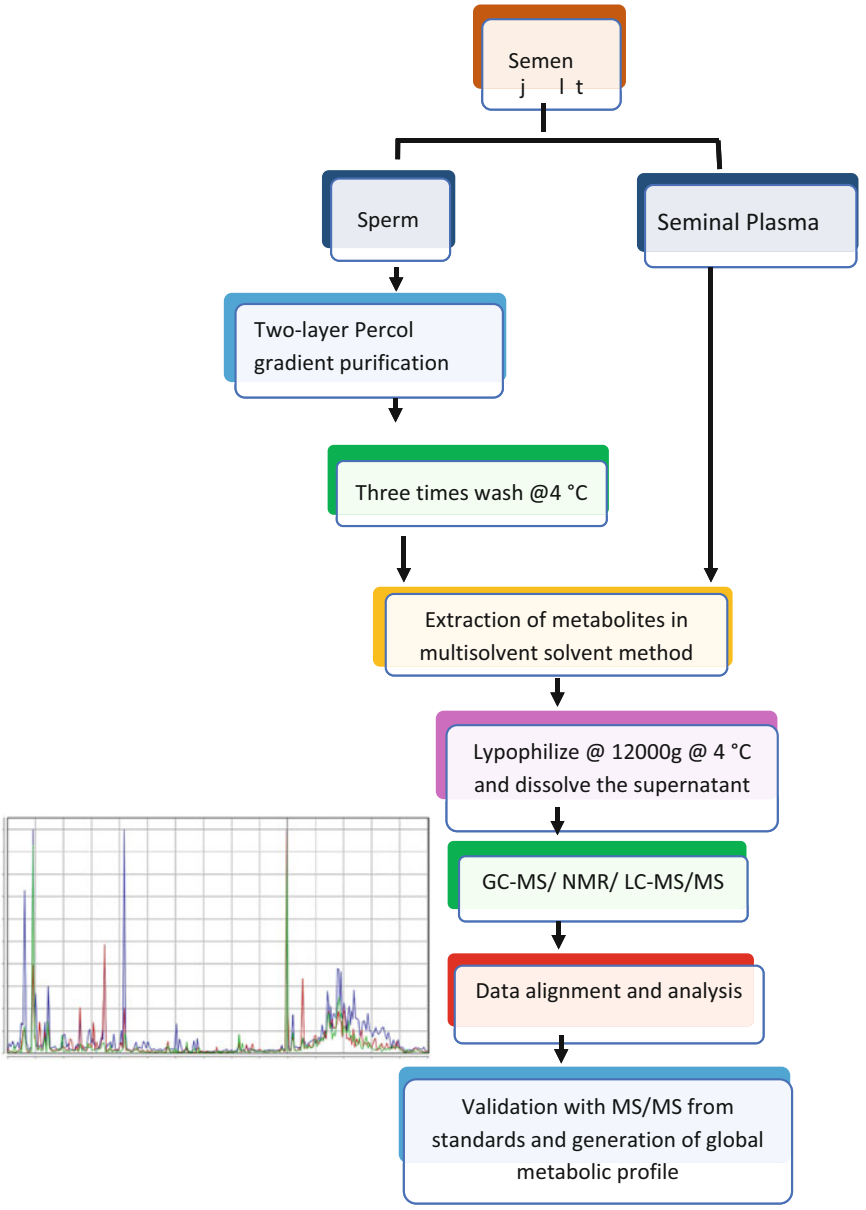

Fig. 8.3 The workflow of semen metabolomics

et al. 2012). Moreover, Ballester et al. (2000) noted the presence of glycogen in head and mid-piece region of canine sperm regulating sperm survival in vivo and controlling sperm capacitation under hypoglycemic condition. Citrulline is an important intracellular source of L arginine, whose conversion to amino acid releases

Table 8.2 Classification of identified of metabolites based on HMDB and PubChem IDs

Class	CAS number	Formula	Match	HMDB ID	Retention time	Mass
Aliphatic acrylic compound						
Carnitine	541–15-1	$C_7H_{15}NO_3$	L-carnitine	HMDB00062	10.24	117
2-aminoethan-1-ol	9007-33-4	C2H7NO	Ethanolamine	HMDB00149	10.13	174
Amino acids, peptides, and analogues						
Serine	302–84-1	C3H7NO3	L-serine	HMDB00187	10.61	116
L-threonine	72–19-5	C4H9NO3	L-threonine	HMDB00167	11.04	130
Glycine	56–40-6	C2H5NO2	Glycine	HMDB00123	11.222	174
Carbohydrates and carbohydrate conjugates						
d-ribose	50–69-1	C5H10O5	D-ribose	HMDB00283	15.71	217
Lipids						
Cholesterol	57–88-5	C27H46O	Cholesterol	HMDB00067	28.29	129
Desmosterol	313–04-2	C27H44O	–	HMDB02719	28.60	129
Palmitoleic acid	373–49-9	C16H30O2	Palmitoleic acid	HMDB03229	34.47	129
Nucleosides, nucleotides, and analogues						
Uridine-5′-monophosphate	58–97-9	C9H13N2O9P	Uridine 5′-monophosphate	HMDB00288	27.36	169
Organic acids and derivatives						
2-Ketoglutaric acid	328–50-7	C5H6O5	Oxoglutaric acid	HMDB00208	17.54	173
Other/unknown						
Phosphoric acid	1071-23-4	C2H8NO4P	O-Phosphoethanolamine	HMDB00224	16.87	174

nitric oxide, a known vasodilator that plays multiple functions in female and male reproduction (Rosselli et al. 1998). On the other hand, fatty acid is found to interact with the integral components of triglycerides, ketone bodies, and phospholipids which takes an active part in generation of energy and formation of lipid bilayer of sperm. Phosphatidylcholine, phosphatidylethanolamine, and PS (16:0/16:0) are the major membrane lipid of sperm involve in membrane-based cascade such as capacitation, acrosome reaction, and sperm-oocyte fusion. These lipid bilayer membranes undergo sensational changes during epididymal maturation and facilitate efflux of cholesterol and phospholipid between sperm and seminal plasma (Scolari et al. 2010; Lucio et al. 2017). Besides, the contents of several amino acids (alanine, histidine, and phenylalanine), citrate, lactate, and GPC in seminal plasma are found to alter in astheno-oligozoospermia patients. Moreover, lysine is suggested as a potential biomarker for the detection and diagnosis of idiopathic infertility (Jayaraman et al. 2014). However, the molecular metabolites in the seminal plasma and spermatozoa have the potential to alter the male fertility. There is an empirical association of the metabolites with the male fertility and semen quality, and the identified biomarkers have the potential to disturb the male reproductive status of different species. Some identified metabolites in seminal plasma and spermatozoa are presented in Table 8.3.

8.8 Association of Metabolites with Fertility

Under consideration, with the emergence of metabolomics, extensive study of myriads of low molecular weight metabolites in sperm and seminal plasma has been explored to ascertain its role in male (in)fertility. Metabolomic studies have been performed in sperm cells from different species, such as goat (Patel et al. 1998), boar (Marin et al. 2003), turbot (Dreanno et al. 2000), and rhesus macaque (Hung et al. 2009). Rat testicular tissue (Griffin et al. 2009) and human seminal plasma have also been subjected to metabolomic analyses (Gupta et al. 2013). Kumar et al. (2015) reported low level of citrate and isoleucine and high level of tryptamine, taurine, and leucine in high-fertile bull seminal plasma as compared to low-fertile bulls. Hypotaurine which is the precursor of taurine, an aminosulfonic acid, is abundant in high-fertile bull spermatozoa as compared to low-fertile bull spermatozoa (Saraf et al. 2020). Higher level of cysteine and taurine is found to be associated with the sperm motility and concentration in the seminal plasma of asthenozoospermia patients (Zhang et al. 2015). On the other hand, mutation of valine to isoleucine causes severe male infertility and adversely affects testicular function (Zhang et al. 2007). Higher NO level in asthenozoospermia men semen is (Perera et al. 1996; Balercia et al. 2004) associated with the increased level of ROS and decreased ATP/Ca2+ (Rahman et al. 2011). Basically, nitroprusside is a nitric oxide donor potentially regulates capacitation, acrosome reaction, and male fertility (Buzadzic et al. 2015). Traces of myo-inositol also found to play an intriguing role in maturation and migration of epididymal male gametes (Palmieri et al. 2016). Additionally, 2E-Dodecenoyl-CoA involved in fatty acid beta oxidation, a part of lipid

Table 8.3 Interaction of identified metabolites in seminal plasma and spermatozoa

Metabolites	Association	Function	Reference
Seminal plasma			
Citrate	Direct	Capacitation, acrosome reaction	Kumar et al. (2015)
Tryptamine	Direct	Capacitation	Kumar et al. (2015)
Taurine	Direct	Enhances motility and capacitation	Devi et al. (2009)
Leucine	Direct	Motility, capacitation, and acrosome reaction	Kumar et al. (2015)
Oleic acid	Indirect	Sperm motility and concentration	Zerbinati et al. (2017)
Inorganic phosphate	Direct	Motility and fertilizing capacity of human sperm	Yi et al. (2012)
Benzoic acid	Direct	Sperm hyperactivation, capacitation	Tang et al. (2017)
Carbamate	Direct	Regulate their intracellular pH	Nishigaki et al. (2014)
Glycerophosphorylcholine/ choline	Direct	Sperm damage	Hamamah et al. (1993)
Cysteine	Direct	Scavengers of ROS	Zhang et al. (2015)
Spermatozoa			
Palmitic acid	Indirect	Sperm membrane metabolism	Tang et al. 2017
Pelargonic acid	Indirect	Sperm maturation, capacitation, and acrosome reaction	Parent et al. (1999)
Lactate	Indirect	Sperm motility and capacitation	Darr et al. (2016)
Taurine/ hypotaurine	Direct	Reduces intracellular calcium level, improves sperm motility, viability, membrane integrity, and increases cryo-tolerance level	DasGupta et al. (2021a), Chillar et al. (2012)
Phosphatidylcholine	Direct	Capacitation, acrosome reaction, and sperm-oocyte fusion are membrane-based cascade	Lucio et al. (2017)
Nitroprusside	Direct/ indirect	Motility, viability, and sperm function	Digamber et al. (2016); Rahman et al. (2011)
L- cysteine	Direct	Reducing oxidative stress, chromatin integrity	Jannatifar et al. (2019)
Acetyl CoA	Direct	Energy to support the flagellar movement of sperm	Piomboni et al. (2012)
β-Alanine	Indirect	Metabolic activity of the spermatozoa	Hou et al. (2013)
GABA	Direct	Sperm motility, acrosome reaction, and fertilization	Menezes et al. (2019)

Table 8.4 Important metabolites identified by t tests (threshold 0.05) with log10-transformed p values and their abundance in HF (high fertile) and LF (low fertile) spermatozoa (Saraf et al. 2020)

Sr. No.	Compound name	Log 10(P)	p value	FDR	Abundance
1	Hypotaurine	0.0001	3.8505	0.007477	HF
2	L-malic acid	0.002	2.6636	0.023	HF
3	Deoxyuridine triphosphate	0.003	2.5207	0.02659	HF
4	Selenocystine	0.004	2.3965	0.02659	HF
5	Methacrylyl-CoA	0.012	1.9101	0.04934	HF
6	Formyl-CoA	0.001	3.2053	0.011013	LF
7	Malyl-CoA	0.001	2.9403	0.015201	LF
8	3-Phosphoadenylylselenate	0.004	2.3984	0.02659	LF
9	dUDP	0.01	1.9904	0.047159	LF
10	Lactyl-CoA	0.01	1.885	0.04934	LF

metabolism, closely ties with the production of energy (UniProtKB). Several researchers have also reported higher level of phospholipid in seminal plasma of azoo-oligospermia patients (Sebastian et al. 1987; Gulaya et al. 2001; Lucio et al. 2017). Recently, DasGupta et al. (2021a) performed partial least squares discriminate analysis for LC-MS/MS detected fragments to generate a two-dimensional score plot to visualize a clear demarcation at metabolome level between high- and low-fertile bulls. Hypotaurine, selenocystine, and L-malic acid were abundant in high fertile bulls, whereas D-cysteine and chondroitin 4-sulfate were abundant in low-fertile bulls as compared to high-fertile bulls (Saraf et al. 2020). The top ten metabolites were identified in high-fertile and low-fertile bulls based on fold change analysis and allowed enough scope to understand the altered molecular pathways associated with sperm metabolism between high and low-fertile bulls (Table 8.4). Although alteration in bovine astheno-oligozoospermia sperm and seminal plasma metabolites involving in fatty acid oxidation, phospholipid, and energy metabolism was detected by using LC-MS/MS analysis (DasGupta et al. 2021b), the clear picture of molecular association of identified metabolites with fertility still needs to be elucidated. Table 8.5 provides the list of major metabolites identified in semen and the possible pathways where these metabolites could have an important role.

8.9 Perspective and Prospective

Though there are many reports of identified differentially expressed proteins and biomarker molecules in the sperm and seminal plasma of bulls related to fertility, their practical application in semen evaluation or bull screening is not well established so far. The major hurdle in making use of identified biomarker candidates is the lack of proper validation in a wide population of bulls, corresponding to different breeds and environment. Proper validation is a costly and arduous procedure, which requires field data of several bulls regarding the

Table 8.5 Identified metabolites along with its classification, involved pathways, and action on sperm of different species

Metabolites	Involved pathway	Action	Identified in species	Reference
Glycolytic substrate	Tyrosine phosphorylation and energy production	Sperm motility	Bull	Goodson et al. (2012)
Pyruvate	Tyrosine phosphorylation and energy production	Sperm motility	Bull	Qiao et al. (2017)
Lactate	Energy production	Sperm motility	Bull	Marin et al. (2003)
Sorbitol	Converts to fructose by sorbitol dehydrogenase present in sperm plasma membrane and involve in production of energy	Sperm survival and motility	Bull	Velho et al. (2018)
Fructose	Energy production pathways and also required for ATP production and also increases respiratory activity	Sperm survival and motility	Bull	Ford (2006)
Glycogen	Energy production pathway	Sperm survival and capacitation	Head and mid piece of canine sperm and human	Ballester et al. (2000)
Citric acid	Ph regulator and act as chelator of Zn, Mg, and Ca	Sperm capacitation, acrosome reaction, sperm movement, and fertilization	Bull, boar, human and rabbit	Sørensen et al. (1999)
Lactic acid	Energy production	Sperm motility	Bull and boar	Dills et al. (1981)
Urea	End product of seminal plasma protein degradation	Unknown	Human seminal fluid and bull seminal fluid	Newairy et al. (2009)
Formate	Inhibit cytochrome oxidase and disrupt mitochondrial electron transport and energy production and finally increasing ROS	Decreases motility and survivality	Human sperm	Nicholls (1976)
2-oxaloglutaric acid	Transfer of acetyl group into mitochondria	Sperm membrane injury and poor semen quality	Boar, goat, and bull	

(continued)

Table 8.5 (continued)

Metabolites	Involved pathway	Action	Identified in species	Reference
				Moore and Bedford (1978)
Glycerophosphocholine	Provides a protective coating to the sperm, lipid metabolism pathway, and phospholipid metabolism	Energy source and storage, sperm motility, and sperm respiratory activity	Ram, bull, human	Sane et al. (1982)
Phosphoric acid	Release energy by hydrolyzing inorganic pyrophosphate to two molecules of phosphoric acid	Sperm motility and fertilization	Bull	Yi et al. (2012)
Tryptamine	Tryptophan metabolism	Promotes acrosome reaction and regulate motility in capacitated sperm	Hamster	Jimenez-Trejo et al. (2012)
Taurine	Antioxidant defense system and by scavenging radical protect the sperm DNA from damage	Increases sperm survivability and post-thaw motility	Bull	Chillar et al. (2012)
Isoleucine and leucine	Reduce calcium uptake by the ejaculated sperm and alter calcium transport across the plasma membrane	Sperm motility, capacitation, and acrosome reaction	Bull seminal plasma	Kumar et al. (2015)
Glutamic acid	Amino acid metabolism in unpredicted male infertility and control ammonia production by converting various amino acid into urea and glutamine. Urea is eliminated as urine, and glutamine is not toxic	Male reproduction	Bull	Qiao et al. (2017)
Carnitine	Transfer fatty acyl group across the inner mitochondrial membrane, regulate B-oxidation	Energy metabolism, maturation, sperm motility, anti-apoptotic and antioxidant factors	Human sperm	Amaral et al. (2013)
Tyrosine	Precursor of neurotransmitter, i.e., adrenalin	Sperm motility	Stallion	Urra et al. (2014)

Acetyl carnitine	Lipid metabolism, fatty acid oxidation, and energy production in spermatogenesis	Sperm motility and antioxidant actions	Human sperm	Amaral et al. (2013)
Stearic acid	Energy metabolism	Sperm motility and viability	Canine, bull seminal plasma	Ortega-Ferrusola et al. (2009)
Docosahexaenoic acid	Regulate membrane fluidity	Sperm motility	Chicken, human and rhesus monkey	Conquer et al. (1999)
Hexadecanoic acid/ palmitic acid	Fatty acid metabolism and lipid metabolism pathway	Energy source, membrane stabilizer, acrosome reaction and fertilization	Canine, bull and human seminal plasma	Velho et al. (2018)
Oleic acid	Energy and phospholipid metabolism in sperm membrane	Sperm motility, viability, and acrosome reaction in pig	Canine, pig, human seminal plasma	Tang et al. (2017)
Cholesterol	Protein tyrosine phosphorylation and PKA activation	Inhibit capacitation, sperm motility, and fertilization	Spermatozoa and seminal plasma of bull	Cross (1996)
Desmosterol	Intermediate in the synthesis of cholesterol	Increases membrane fluidity and motility and potent inhibitor of capacitation	Spermatozoa of bull, human, monkey and hamster	Connor et al. (1998)
Cholesterol sulfate	Accumulates in spermatozoa during epididymal transit, most specifically in the acrosome head region and acts as membrane stabilizer	Inhibit capacitation and potent inhibitor of Acrosin	Spermatozoa and seminal plasma of bull	Roberts et al. (1988)
Putrescine	Precursor of spermine and spermidine	Involve in spermatogenesis and act as a growth factor in cell division, embryogenesis and increases sperm motility	Human and bull sperm	Morales et al. (2003).

fertility or conception rate in cows. Moreover, the lack of consistency in identified biomarker molecules among various studies also makes the validation difficult. However, certain molecules like BSP, ENO1 which are consistent among various studies can be adopted as biomarker candidates if proper validation can be performed using advanced techniques like MRM.

Metabolomics in reproduction is relatively an emerging area with meager information about the identified metabolites and application of LC/MS technique in different fertility status of an individual as well as in domestic livestock species. This technology has introduced variability in analysis of linear decline in semen quality at molecular level and predicted biomarkers in sperm cells of different species such as bull, goat, boar, stallion, and human. However, metabolome database is under monotonous development, and identification of metabolites along with different concentration and altered metabolic pathways in clinical diagnosis has become a challenging to the diagnostic and treatment of male infertility. This novel approach has thrown light towards elucidating the hidden reasons of average semen production period and higher incidence of poor seminal attributes in crossbred bull sperm and seminal plasma. Yet the full potential of metabolomics is not explored due to the limitation of existing database. Thus, in future days, metabolites can illuminate a deep insight into a clinical biomarker for therapeutic and diagnostic in infertility cases.

Considering the huge loss incurred for rearing unproductive bulls, directly and indirectly, it is high time that proper methods for screening the expression of identified biomarker candidates should be incorporated with routine semen evaluation techniques. Since the fertilizing ability of spermatozoa depends upon several factors and molecules, rather than going for a single molecule, a panel of biomarkers may be identified and used for the screening or evaluation. Besides, through innovative approaches, if the fertility biomarkers can be identified at an early stage especially in testicular cells, it will be of great help in identifying and removing the inferior quality bulls at an early stage, thereby saving valuable resources.

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Spermatogonial Stem Cells: Their Use in Fertility Restoration

9

Ankur Sharma, A. Kumaresan, Preeti Vats, and Manmohan Singh Chauhan

Abstract

Spermatogonial stem cells (SSCs) are the only adult stem cells in males capable of transmitting their genetic information to the next generation. Over the past two decades, spermatogonial stem cell transplantation has shown colossal potential for fertility restoration and transgenesis in livestock. It is based on the transfer of SSCs from fertile donors or genetically modified cells into the testes of suitable homologous recipients, which confer them the ability to produce donor SSCs derived spermatozoa. After its efficient implementation in rodents, attempts are underway to adopt this technique for livestock as a novel tool for infertility treatment and transgenic animal production. Although the research in this area in livestock species is still at the infant stage, preliminary studies have shown remarkable success. There is additional room for significant improvement in methods for SSC enrichment, long-term in vitro propagation, and endogenous germ cell depletion to improve the offspring production rate using this method. This chapter provides a basic understanding and current status of the SSC transplantation technique in livestock and its potential application for fertility restoration in these species.

A. Sharma (✉)
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

Animal Biotechnology Centre, ICAR-National Dairy Research Institute, Karnal, Haryana, India

A. Kumaresan
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

P. Vats · M. S. Chauhan
Animal Biotechnology Centre, ICAR-National Dairy Research Institute, Karnal, Haryana, India

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Keywords

Fertility · Livestock · Spermatogonial stem cell · Transplantation · Transgenesis

9.1 Introduction

Spermatogenesis is a highly orchestrated cascade of events that maintain male fertility by continuously generating millions of spermatozoa every day throughout the adult life of males. This productive system relies upon spermatogonial stem cells (SSCs), the unipotent adult stem cells of the testis that reside in a specialized microenvironment at the basement membrane of seminiferous tubules. The SSCs perpetually self-renew and simultaneously generate many daughter cells committed to differentiation into spermatozoa. The balance between self-renewal and differentiation is under meticulous quality control to safeguard germline integrity. SSCs bear the unique capacity to pass on their genetic information to the upcoming generations that have encouraged the scientific community to explore their application in infertility treatment and generating transgenic animals. In this regard, the SSC transplantation technique, which involves the injection of functional SSCs harvested from fertile donors into infertile recipient testes, could play a crucial role. Upon transplantation, the donor's SSCs colonize the "stem cell niche" in the recipient's seminiferous tubules and, after that, generate donor-derived functional spermatozoa. Pioneering studies in the mouse model have demonstrated that transplantation of functional SSCs in infertile recipients restored their fertility and conferred the ability to produce live offspring (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). Subsequently, this technique was successfully used for generating transgenic mice (Brinster and Avarbock 1994; Nagano et al. 2001) and rats (Hamra et al. 2002; Ryu et al. 2007). The promising results in rodents have laid the foundation for the application of SSC transplantation in livestock. So far, this technique has been optimized in various farm animal species with varying success (Honaramooz and Yang 2011; Sharma et al. 2020a, c). Live offspring production has been reported in goat (Honaramooz et al. 2003a) and sheep (Herrid et al. 2009a). In the future, SSC transplantation holds the potential to play a crucial role in restoring fertility in livestock, preserving and propagating the animals of high genetic merit or endangered species. For instance, it could address the infertility/subfertility problem in crossbred males in India, which causes massive economic loss to the livestock sector. Additionally, it can serve as an efficient alternative for generating transgenic farm animals while offering higher efficiency and less time and capital input.

9.2 Origin of SSCs

SSCs arise from gonocytes in the postnatal testis, whereas the gonocytes originate from primordial germ cells (PGCs) during fetal development. In murine embryonic development, the PGCs are first visible at the epiblast stage embryo 1 week post

coitum (Phillips et al. 2010). After that, these cells migrate to the genital ridge via hindgut between 8.5 and 12.5 days post coitum (dpc), during which they proliferate, and ultimately, ~3000 cells colonize the genital ridge (Bendel-Stenzel et al. 1998). In the male gonad, PGCs differentiate into gonocytes at ~13.5 dpc, which then get enclosed in the center of testicular cords formed of precursors of peritubular myoid cells and Sertoli cells. The gonocytes proliferate until 16.5 dpc and are arrested at the cell cycle's G0/G1 stage (McLaren 2003). These cells resume proliferation after 1 week of birth and concomitantly migrate to the basement membrane of seminiferous tubules and transform into SSCs. Among livestock, the transformation of gonocytes into SSCs occurs after ~2 months of birth in goats and pigs, whereas ~3 and 4 months in sheep and cattle, respectively (Zheng et al. 2014a).

The mechanism of SSC proliferation varies slightly between non-primate and primate mammals. Among the non-primates, the A_{single} (A_s) model for SSC self-renewal is prevalent (Oakberg 1971; Huckins 1971). Among the different subtypes of undifferentiated spermatogonia, it considers only A_s spermatogonia as the "true" SSCs. The A_s spermatogonia proliferate, and approximately half of the cells undergo self-renewing division and generate two distinct A_s cells to maintain the stem cell pool. In contrast, another half of A_s cells divide and produce two daughter cells interconnected by cytoplasmic bridges, known as A_{paired} (A_{pr}) cells. The A_{pr} spermatogonia further generate four interconnected cells known as $A_{aligned}$ (A_{al}) spermatogonia. The following divisions of A_{al} spermatogonia produce 8, 16, and occasionally 32 interlinked cells. Subsequently, the A_{al} spermatogonia undergo morphological changes and transform into type A_1 spermatogonia, which are the first step towards the generation of differentiating type A spermatogonia. Contrarily to this model, the recent findings have elucidated that during the regeneration of damaged testes, the A_{pr} and A_{al} cells regain the stem cell activity to resume the spermatogenesis (Nakagawa et al. 2007).

Among primates, there are two morphologically distinct subclasses of type A spermatogonia, viz. A_{dark} (A_d) and A_{pale} (A_p). The A_d spermatogonia remain undifferentiated and serve as regenerative reserve stem cells which do not divide, whereas the A_p spermatogonia play a progenitor role and serve as a functional reserve (Ehmcke et al. 2005). The self-renewal mechanism of A_p spermatogonia is comparable to the A_s model as they persist not only in single-cell form but also as clones of 2, 4, and 8 cells.

9.3 SSC Niche Regulate Their Self-Renewal

The adult stem cells reside in a specialized microenvironment known as the "stem cell niche" that regulates their self-renewal and differentiation to maintain tissue homeostasis. SSCs inhabit the specialized niche microenvironments at the basement membrane of seminiferous tubules. The SSC niche comprises SSC, encompassing Sertoli cells, peritubular myoid cells, Leydig cells, and vasculature. SSC self-renewal and differentiation regulation is highly complex and regulated by various paracrine growth factors and signaling molecules secreted by the niche constituents.

In this regard, Sertoli cells are of foremost importance as being the only somatic cells in direct contact with the SSCs and differentiating germ cells. These cells provide glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF), essential for SSC self-renewal. GDNF is the prime growth factor crucial for SSC self-renewal both in vitro and in vivo, whereas bFGF plays a supporting role and cannot support their maintenance in culture to a full extent independently (Kubota et al. 2004a). Additionally, the colony-stimulating factor-1 (CSF1) produced by Leydig cells and testicular macrophages and vascular endothelial growth factor (VEGF) by vasculature have also shown a positive effect on the proliferation of cultured SSCs, which suggest their possible role in determining the fate of cultured SSCs. However, it is yet to be studied in detail.

9.4 SSC Transplantation for Fertility Restoration

The major causes of male infertility include aberrant spermatozoa production/function, impaired delivery of spermatozoa, and overexposure to certain environmental gonadotoxins. The pathogenesis of male infertility can be ascribed to the disorders related to the SSC proliferation and differentiation or somatic cell dysfunction (Vlajković et al. 2012). Other infertility causes include cryptorchidism, testicular cancer, and chemotherapy and radiotherapy for cancer treatment. In cases where infertility is caused because of the germ cell defect, SSC transplantation could play a key role in fertility restoration in these animals. In 1994, for the first time, Brinster and colleagues successfully restored the fertility in mice by injecting the SSCs harvested from fertile donors into infertile recipients (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). Following the transplantation, the donor SSCs could successfully generate fully mature and functional spermatozoa, and after that, live offspring were produced after natural mating. After that, Ogawa et al. (2000) restored the fertility in infertile mice having germ cell defects via injecting merely $<$200 SSCs. This number was even less than 1% of the total SSCs usually present in a wild-type testis (Brinster 2002), demonstrating the enormous regenerative caliber of this technique. In the following several studies, infertility caused due to germ cell or Sertoli cell defects was cured by the transplantation of wild-type or genetically modified donor cells (Kanatsu-Shinohara et al. 2003a; Takehashi et al. 2007; Ciccarelli et al. 2020). Later on, this method was efficiently adopted in rats, and the SSC transplantation among genetically unrelated animals was able to establish spermatogenesis and produce donor-derived offspring (Jiang and Short 1995; Ogawa et al. 1999).

The initial triumph in rodents has provided a gateway for the potential application of this technique for biomedicine and agriculture science in humans and livestock. In humans, the use of chemotherapy or radiotherapy for cancer treatment could adversely affect patients' fertility via depleting their SSCs. The adult males have an option to safeguard their germplasm via cryopreserving their semen before undergoing the oncological treatment although with a limited success (~25% success rate; Blackhall et al. 2002). However, this method is not feasible to preserve fertility

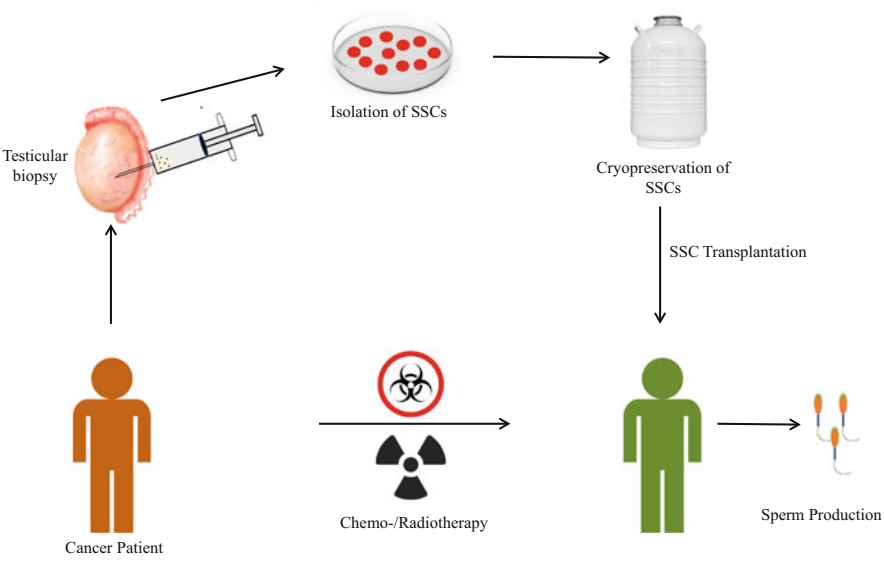

Fig. 9.1 A schematic representation of fertility restoration in patients undergoing chemo-/radiotherapy by SSC transplantation

in prepubertal boys since they do not produce mature spermatozoa at this stage. In such cases, as illustrated in Fig. 9.1, SSCs can be harvested from a testicular biopsy and cryopreserved prior to the chemo- or radiotherapy. After eliminating malignant cells, SSCs could be thawed and reintroduced into the patient's testes to restore their fertility. The first clinical trial for autologous SSC transplantation in lymphoma patients was reported in 1999 (Radford et al. 1999). Further advancements in optimizing culture conditions for human SSCs and their in vitro differentiation into round/elongated spermatids for intracytoplasmic sperm injection (ICSI) would revolutionize the clinical prospects of germ-cells-based therapy in humans.

Over the past two decades, SSC transplantation has been attempted in various farm animal species, including pigs (Honaramooz et al. 2002; Mikkola et al. 2006; Zeng et al. 2013; Kim et al. 2014), goats (Honaramooz et al. 2003a, b, 2008), rams (Rodriguez-Sosa et al. 2006; Herrid et al. 2009a, 2011), cattle (Izadyar et al. 2003; Herrid et al. 2006; Stockwell et al. 2009), buffalo (Sharma et al. 2020a, c), and dromedary camel (Herrid et al. 2019) with varying success. The SSC transplantation in rodents requires inherently immune-deficient or genetically compatible hosts to the donors; otherwise, immunosuppressive drugs can also be used for allogeneic transplantation (Kanatsu-Shinohara et al. 2003a; Zhang et al. 2003). However, in farm animals, the donor SSCs from genetically unrelated donors were tolerated in homologous recipients with an intact immune system (Honaramooz et al. 2003a; Herrid et al. 2006; Zeng et al. 2013; Kim et al. 2014; Sharma et al. 2020a, c). This unique feature ensures this technique's feasibility and field applications in livestock. In India, subfertility and poor semen freezability are the significant reasons for a high

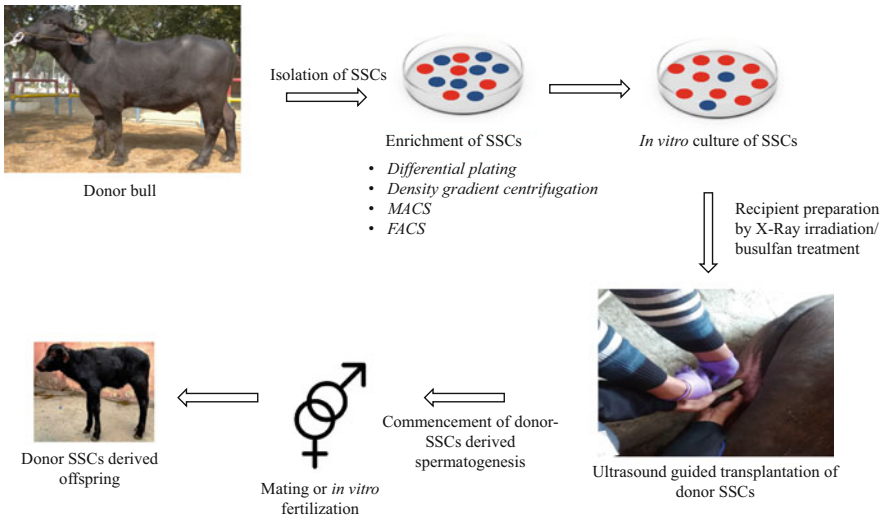

Fig. 9.2 A schematic representation of steps involved in SSC transplantation

culling rate (40–70%) in crossbred males, which eventually hampers the availability of superior germplasm for the breeding program (Kumaresan et al. 2021). The optimization of efficient in vitro SSC propagation and subsequent transplantation procedures for these animals could assist in overcoming these problems. The various steps involved in SSC transplantation are illustrated in Fig. 9.2.

9.5 Isolation and Enrichment of SSCs

The SSCs are scarce in the testis, for instance, ~0.03% in adult mice testis (Tagelenbosch and de Rooij 1993); therefore, their isolation with high purity and viability is of paramount importance for their use in genetic manipulation and transplantation. The protocols for SSC isolation vary among laboratories depending on the species and the target cell type (SSC/gonocyte). However, the two-step enzymatic digestion is the prevalent method for isolating gonocytes and SSCs from different species. In this method, hyaluronidase and collagenase eliminate the interstitial cells, whereas trypsin-EDTA breaks down the seminiferous cords/tubules to release the SSCs. DNase I is added to prevent cellular aggregation. SSCs/gonocytes could be isolated with up to 10% purity (Honaramooz and Yang 2011). In further developments, a novel approach termed a "three-step strategy" was developed wherein the combined use of vortexing and gentle enzymatic digestion could yield the porcine gonocytes with ~40% purity (Yang et al. 2010).

The freshly isolated testicular cell suspension is usually subjected to an enrichment step to improve the purity of SSCs further. For this, numerous approaches have been used, such as differential plating, density gradient centrifugation,

fluorescence-activated cell sorting (FACS), and magnetic-activated cell separation (MACS). Differential plating and density gradient centrifugation enrich the SSCs based on their physical properties, whereas FACS and MACS rely upon fluorophore-labeled antibodies directed against the surface markers for SSCs. The freshly isolated cells are incubated with the extracellular matrix (gelatin, collagen, lectin, and laminin) coated culture vessels for the defined time durations in differential plating. Since SSCs bear lower adherent velocity than the somatic cells, therefore tend to remain in suspension while the contaminating somatic cells adhere to the surface of culture vessels. Density gradient centrifugation is another approach for SSC enrichment wherein a density gradient is created using Percoll or Nycodenz. Then the SSCs are separated from the somatic cells based on the difference in their sedimentation velocities due to the variations in their size and mass. The combined use of differential plating and density gradient centrifugation is more effective for SSC/gonocyte enrichment than the methods used alone (Izadyar et al. 2002; Yang and Honaramooz 2011; Ahmad et al. 2013; Sharma et al. 2020a). By combining these two approaches, SSCs and gonocytes could be enriched up to 90% (Yang and Honaramooz 2011; Ahmad et al. 2013). We have recently reported the enrichment of SSCs from the water buffalo by differential plating using *Datura stramonium* agglutinin (DSA) lectin followed by Percoll density gradient centrifugation resulted in an increase of ~2.3-fold in DBA^+ and ~12-fold in $THY1^+$ cells (Fig. 9.3; Sharma et al. 2020a).

The methods mentioned above enable the user to harvest the SSCs in large quantities with high viability, therefore generally preferred for donor cell preparation for transplantation in livestock. However, other advanced approaches such as FACS and MACS that recognize the molecular phenotypes of the cells are also being explored to enrich SSCs from large domestic animals. For this, surface markers such as DBA, THY1, and SSEA1 have been commonly used. For bovine SSC enrichment, the use of FACS resulted in a four-fold increase in DBA^+ cells, whereas, using MACS, up to five-fold enrichment was achieved (Herrid et al. 2009b). Another study revealed that among the MACS enriched bovine SSCs using THY1, 64.4% were also positive for PLZF expression (Reding et al. 2010). THY1 based MACS could yield highly enriched SSC populations in pigs (Zeng et al. 2013) and goats (Abbasi et al. 2013). Compared to the FACS, MACS is a relatively simple and cost-effective method that does not require much technical expertise and provides reproducible results. It also holds the potential for scale-up to harvest the large SSC population for transplantation in livestock.

9.6 Characterization of SSCs

The isolated SSCs are characterized based on the expression of specific biochemical markers prior to their in vitro propagation and further downstream application in transplantation and genetic manipulation. Recently, numerous molecular markers have been identified for SSCs in rodents although none of these markers is specific to the A_s spermatogonia and instead are conserved among all three subtypes of

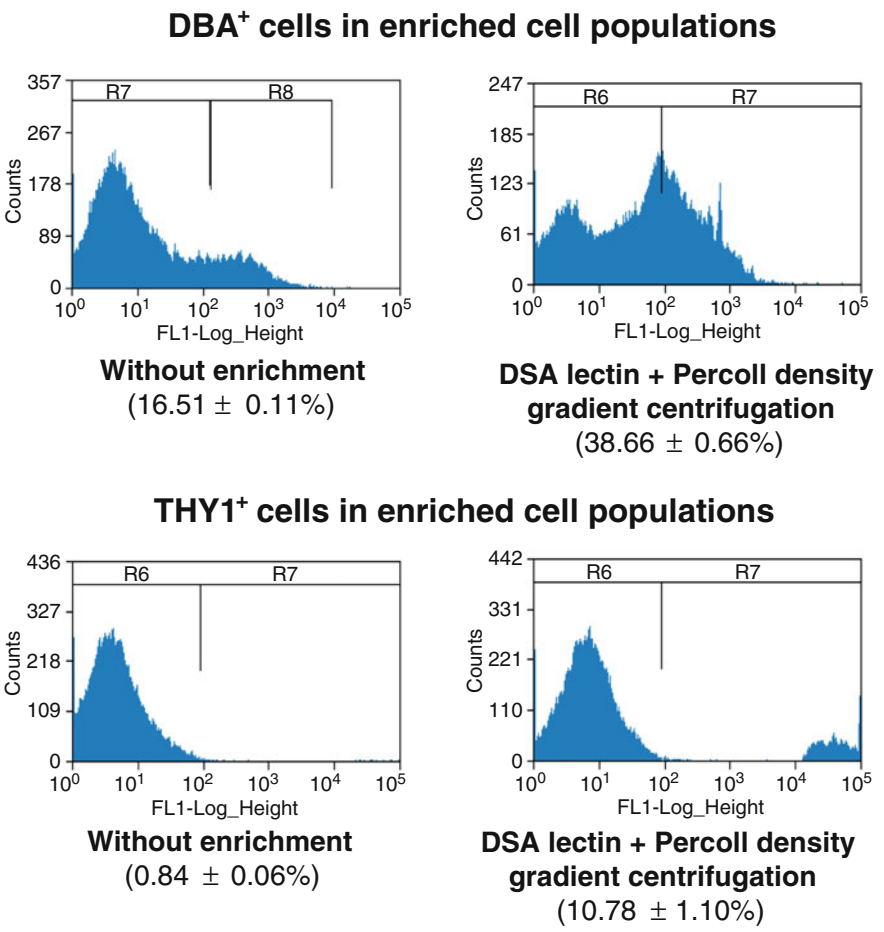

Fig. 9.3 Flow cytometry based evaluation of SSC enrichment by differential plating with *Datura stramonium* agglutinin (DSA) lectin followed by Percoll density gradient centrifugation using DBA and THY1 as markers. Values are mean ± SEM. (Adapted from Sharma et al. 2020a)

undifferentiated type A spermatogonia (A_s, A_{pr}, and A_{al}). Therefore, a battery of markers is currently used to confirm their bona fide stem cell identity (Fig. 9.4; Table 9.1). These markers include alkaline phosphatase activity, ID4, UCHL1, PLZF, GFRα1, α6 integrin, β1 integrin, THY1, DBA, CD9, c-kit, OCT4, etc. SSC transplantation is the only in vivo functional bioassay available to confirm their stem cell potential wherein SSCs are transplanted into homologous recipients or in nude mice as a universal recipient model. The donor SSCs transplanted into homologous recipients can colonize the seminiferous tubules and establish fully functional spermatogenesis. However, in xenotransplantation, the donors' SSCs undergo proliferation but cannot establish complete spermatogenesis.

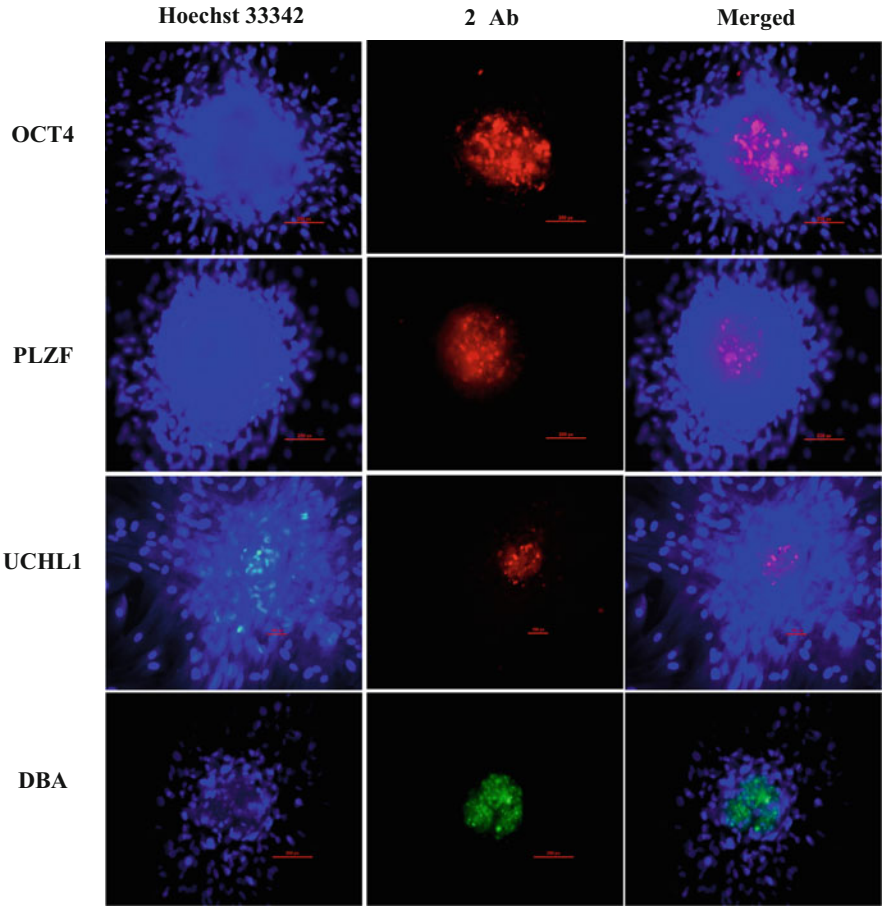

Fig. 9.4 Immunocytochemical analysis of spermatogonial markers OCT4, PLZF (200×; 200 μm); UCHL1 (100×; 100 μm); and DBA (100×; 200 μm) in putative SSC colonies on 12th day of culture. Hoechst 33342 nuclear stained image (column A), secondary antibody labeled image (column B), merged image of column A and B (Column C)

9.7 In Vitro Propagation of SSCs

Given the very limited population of SSCs within the testis, optimizing an efficient culture system capable of supporting their long-term self-renewal is critical to ensure their sufficient number for use in transplantation and genetic manipulation. To date, long-term SSC culture has been established for mice, rats, and hamsters (Kanatsu-Shinohara et al. 2003b, 2008; Kubota et al. 2004b; Ryu et al. 2005), whereas, for livestock, the SSCs could be maintained for short duration only (usually up to 2 months; Aponte et al. 2006, 2008; Goel et al. 2007, 2009; Kuijk et al. 2009; Bahadorani et al. 2012; Sharma et al. 2019a, 2020b). The culture conditions used for

Table 9.1 Overview of commonly used molecular markers to identify SSCs from livestock species

Markers	Species				
					
DBA	+ (Sharma et al. 2020b)	+ (Borjigin et al. 2010)	+ (Goel et al. 2007)	+ (Izadyar et al. 2002)	+ (Sharma et al. 2020a)
THY1/ CD90	+ (Abbasi et al. 2013; Sharma et al. 2020b)	+ (Bahadorani et al. 2011)	+ (Zheng et al. 2014b)	+ (Reding et al. 2010)	+ (Sharma et al. 2020a)
UCHL1/ PGP9.5	+ (Sharma et al. 2020b)	+ (Rodriguez-Sosa et al. 2006)	+ (Luo et al. 2006)	+ (Herrid et al. 2007)	+ (Sharma et al. 2019a, 2019b)
PLZF	+ (Sharma et al. 2020b)	+ (Borjigin et al. 2010)	+ (Luo et al. 2006)	+ (Reding et al. 2010)	+ (Sharma et al. 2019a, 2019b)
OCT4	+ (Sharma et al. 2020b)	+ (Qasemi-Panahi et al. 2018)	+ (Goel et al. 2008)	+ (Fujihara et al. 2011)	+ (Sharma et al. 2019a, 2019b)
GFRα1	+ (Wu et al. 2013)	N.D.	+ (Lee et al. 2013)	+ (Sahare et al. 2016)	N.D.
VASA/ DDX4	+ (Bahadorani et al. 2011)	+ (Borjigin et al. 2010)	+ (Kim et al. 2013)	+ (Fujihara et al. 2011)	+ (Goel et al. 2010)
NANOG	+ (Sharma et al. 2020b)	+ (McLean et al. 2019)	+ (Goel et al. 2008)	+ (Fujihara et al. 2011)	+ (Sharma et al. 2019a, 2019b)

+ positive results, *N.D.* not determined

SSCs from rodents could not support the proliferation of those from livestock beyond the primary culture stage. It indicates that these cells may hold specific unique requirements yet to be explored. Attempts are underway to optimize the culture conditions for SSCs from livestock species, emphasizing the selection of optimum culture medium, identifying appropriate growth factor regimes, and developing serum- and feeder-free culture conditions.

The use of serum-supplemented DMEM and DMEM/F12 has been an integral part of SSC culture across the species. However, the inclusion of serum in culture

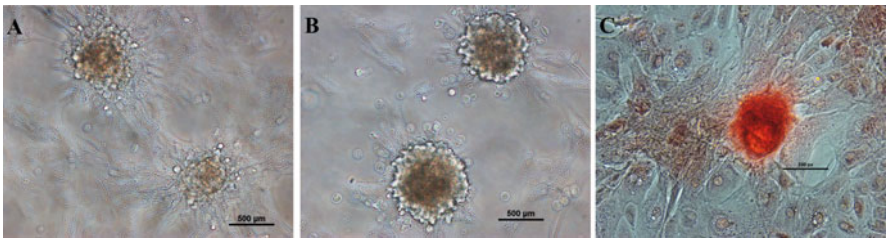

Fig. 9.5 In vitro culture of buffalo putative SSCs in Ko-DMEM + 10% KOSR. (**a**) Putative SSC colonies as observed on 7th day of culture (200×; 500 μm); (**b**) 15th day of culture (200×; 500 μm); (**c**) Alkaline phosphatase staining of putative SSCs (400×; 200 μm). SSC, Spermatogonial stem cell

medium introduces specific undefined complex parameters that exert detrimental effects on SSC self-renewal and colony formation (Kubota et al. 2004a). Therefore, efforts have been made to explore serum-free culture media as an alternative. In this regard, the StemPro medium is adopted for the prolonged culture of SSCs from mice (Kanatsu-Shinohara et al. 2003b) and hamsters (Kanatsu-Shinohara et al. 2008), and recently, it was also tested for pigs (Kuijk et al. 2009) and bulls (Aponte et al. 2008). We have recently reported that the use of KO-DMEM supplemented with 10% KOSR for the propagation of goat (Sharma et al. 2020b) and buffalo (Fig. 9.5; Sharma et al. 2019a, b) SSCs promoted their self-renewal, and the colonies could be maintained for a longer duration than those cultured in a serum-supplemented medium (Sharma et al. 2016).

GDNF and bFGF in supporting SSC self-renewal have been studied in detail. However, there could be variations in their dose requirements among different species. Apart from GDNF and bFGF, other essential growth factors such as LIF, EGF, CSF1, and VEGF have also been reported to exhibit a beneficial effect on in vitro propagation of SSCs and colony formation although their specific roles are yet to be assessed in depth (Oatley et al. 2009; Caires et al. 2009; Tian et al. 2016; Sharma et al. 2019a, b). We also found that the combined use of GDNF, bFGF, LIF, and CSF1 could support buffalo SSC self-renewal better than GDNF alone or GDNF in combination with bFGF (Sharma et al. 2019a).

Feeders are an integral part of SSC in vitro culture wherein mitotically inactivated autologous/homologous Sertoli cells are used since they naturally provide GDNF and bFGF. However, these cells secrete various other growth factors, including those promoting SSC differentiation (Kubota et al. 2004b). Therefore, feeder-free culture conditions using extracellular matrix components such as laminin need to be developed to establish the prolonged SSC culture for livestock species.

9.8 SSC Transfer Techniques

The method used for SSC transplantation in mice involves direct injection of the donor SSCs into a single efferent duct emerging from the rete testis, which is not feasible in the case of livestock due to the anatomic limitations since several efferent

ducts emerge from the centrally located rete testis. Therefore, Schlatt et al. (1999) developed the ultrasound-guided cannulation of rete testis to infuse the donor SSCs according to the gravity flow. This method has been successfully adopted for SSC transplantation in pigs (Honaramooz et al. 2002), goats (Honaramooz et al. 2003b), rams (Herrid et al. 2009a), cattle (Izadyar et al. 2003), buffalo (Sharma et al. 2020a, c), and dromedary camel (Herrid et al. 2019). The ultrasound-guided transplantation procedure under general anesthesia and aseptic surgical conditions could be completed in 15–30 minutes. The donor SSCs are generally injected at the flow rate of ~0.5–1 ml/minute, which fills approximately half of the recipient's seminiferous tubules with the donor SSCs.

9.8.1 Selection of Donors

Researchers have given preference to the prepubertal animals while selecting the donors for SSC transplantation. The prepubertal animals lack the commencement of fully functional spermatogenesis; therefore, they naturally provide a very high population of SSCs. Likewise, gonocytes are the only germ cell type present in the neonatal testis constituting 1–2% of total isolated testicular cells in rodents or approximately 7% of the intra-tubular cells in piglets (Honaramooz and Yang 2011).

9.8.2 Preparation of Recipients

Preparation of suitable recipients that allow the donor SSCs to colonize and restore sperm production is crucial for SSC transplantation. Unlike rodents, for the SSC transplantation in livestock, researchers have given preference to the prepubertal recipients because their testes provide a more favorable environment for engraftment and expansion of donor SSCs than adults (Honaramooz and Yang 2011). Moreover, their seminiferous tubules lack the multiple hindering layers of differentiating germ cells that facilitate easy access of the donor SSCs to colonize the stem cell niche at the basement membrane. The unique feature of SSC transplantation in livestock is that the donor SSCs from genetically unrelated males are immunologically tolerated in the homologous recipient testes. Contrastingly, similar attempts in rodents resulted in limited colonization of recipient testes unless the immunosuppressive drugs were used (Kanatsu-Shinohara et al. 2016). The exact reason for this immune tolerance could not be identified to date although it is believed that the immunomodulatory functions of Sertoli cells might play a crucial role in this phenomenon (Savvulidi et al. 2019). An earlier study has demonstrated the successful SSC transplantation from *Bos taurus* donors to the *Bos indicus* cross recipients (Herrid et al. 2006).

9.8.3 Depletion of Recipient's Endogenous SSCs

The recipient preparation for SSC transplantation in rodents involves the depletion of the recipient's endogenous germ cells to facilitate the availability of stem cell niche to the donor SSCs. It is generally accomplished using busulfan, an alkylating agent that induces apoptosis in rapidly proliferating cells. Contrastingly, in livestock, the recipient preparation by ablation of endogenous SSCs is not a prerequisite for efficient transplantation. Successful SSC transplantation without germ cell depletion has been reported in goats (Honaramooz et al. 2003a, b), pigs (Honaramooz et al. 2002; Zeng et al. 2013), cattle (Herrid et al. 2006), and buffaloes (Sharma et al. 2020a, c). However, if this technique has to be applied as a breeding tool, the depletion of the recipient's endogenous SSCs would be crucial to avoid the production of mixed populations of spermatozoa originating from SSCs of both donor and recipient origin (Oatley 2018). Busulfan treatment and local testicular irradiation are the commonly used approaches for abating endogenous SSCs in livestock. Since the busulfan treatment does not require any sophisticated instrument, therefore, appears to be more applicable in the field conditions, although the systemic toxicity and biohazard risks are the primary concerns related to its use.

Additionally, optimizing the adequate dose of busulfan is critical, as, at the higher dose, it can also damage the Sertoli cells and eventually the stem cell niche. In a recent study from our group, the intravenous dose of busulfan was optimized to efficiently deplete endogenous SSCs in crossbred cattle and pigs (Kumar 2018). 3 mg/kg body weight dose of busulfan was found to be safe for the recipients and could induce a moderate level of depletion of endogenous SSCs in both species.

The local testicular irradiation is the most preferred approach currently used to deplete endogenous SSCs in livestock. However, it requires a costly and sophisticated radiation source. The success of this approach is governed by the following two parameters, viz. dose of irradiation and the age of recipient at the time of treatment. Here also, the radiation dose needs to be optimized as often the higher dose (>5–6 Gy) required for the ablation of SSCs across the species compromises the viability of Sertoli cells and occasionally results in testicular atrophy (Savvulidi et al. 2019).

9.8.4 Recent Advancements in Improving the Efficiency of Transplantation

Considering the limitations of the current approaches for SSC depletion, attempts are underway to find efficient alternatives to the busulfan administration and testicular irradiation to improve the efficiency of SSC transplantation. Herrid et al. (2019) have recently demonstrated that the administration of 25–50 μg/ml DBA via rete testis exhibits selective toxicity to the spermatogonia and efficiently depletes the endogenous SSCs in dromedary camel.

The methods used for ablation of endogenous SSCs often exert detrimental effects on the viability and functionality of Sertoli cells and Leydig cells; therefore,

in addition to the donor SSCs, the co-transplantation of functional Sertoli cells and Leydig cells from homologous donors could reinstate the damaged niche and thereby improve the colonization efficiency of the transplanted SSCs. Moreover, the co-transplantation of SSCs with mesenchymal stem cells has also improved the transplantation efficiency in the mice model (Kadam et al. 2018). Currently, particular emphasis is being given to generating sterile hosts devoid of endogenous germ cells using advanced genome editing tools. Recently, Ciccarelli et al. (2020) have generated genetically sterile male mice, goats, pigs, and cattle bulls by knocking out the *NANOS2* gene using CRISPR-Cas9 technology. These hosts thus created lacked endogenous germ cells, although they were physiologically normal and, upon injection of SSCs from allogeneic donors, could sustain donor-derived sperm production.

9.9 Determining the Fate of Donor SSCs Following Transplantation

As summarized in Table. 9.2, the production of morphologically and functionally competent spermatozoa of the donor origin following SSC transplantation has been evidenced in goats (Honaramooz et al. 2003a, 2008), pigs (Kim et al. 2014), sheep

Table 9.2 Summary of SSC transplantation in livestock animals

Species	Colonization by donor SSCs	Donor SSCs derived spermatogenesis	Offspring	Transgenic progeny
	+ (Honaramooz et al. 2003b)	+	+	+ (Honaramooz et al. 2003a)
	+	+ (Rodriguez-Sosa et al. 2006)	+ (Herrid et al. 2009a)	–
	+ (Honaramooz et al. 2002)	+ (Mikkola et al. 2006)	–	–
	+ (Herrid et al. 2006)	+ (Izadyar et al. 2003)	–	–
	+ (Sharma et al. 2020a, 2020c)	–	–	–
	+	+ (Herrid et al. 2019)	–	–

+ positive results, – negative results.

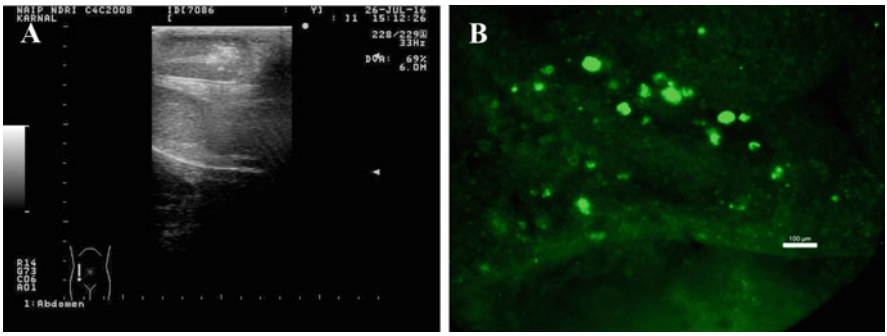

Fig. 9.6 Homologous transplantation of transfected enriched SSCs in the water buffalo. (**a**) Post-transplantation diffusion of donor cells; (**b**) Localization of donor transfected enriched SSCs in recipient's seminiferous tubules after 8 weeks of transplantation. (Adapted from Sharma et al. 2020a)

(Herrid et al. 2009a), cattle (Stockwell et al. 2009), and dromedary camel (Herrid et al. 2019). Generally, the monitoring of the fate of donor SSCs within recipient testes is achieved using fluorescent cell labeling dyes although this approach facilitates the tracking of donor cells up to a few weeks only. We could track the fluorescently labeled bubaline SSCs in recipient buffalo testes up to 8 weeks of transplantation (Sharma et al. 2020c). In another approach, microsatellite detection has been used to confirm the donor germplasm in the recipient's spermatozoa (Stockwell et al. 2009; Herrid et al. 2019). Several researchers have used genetically modified SSCs for transplantation research, which facilitates easy detection of the transgene in the recipient's spermatozoa, and the IVF embryos were generated using these sperm (Honaramooz et al. 2003a, 2008; Zeng et al. 2013). Recently, we have reported the persistence of the buffalo transfected SSCs in the homologous recipient testes for up to the eighth week of transplantation based on the GFP expression (Fig. 9.6; Sharma et al. 2020a). The presence of donor germplasm in the recipient's semen has been tracked for at least 2 years in goats (Honaramooz et al. 2008) and cattle (Stockwell et al. 2009), whereas for 5 years in pigs (Zeng et al. 2013) and sheep (Stockwell et al. 2013).

9.10 Production of Live Offspring Following SSC Transplantation

The most credible affirmation of a successful SSC transplantation is the birth of live offspring carrying the donor germplasm. The first study on SSC transplantation in mice demonstrated that some of the infertile recipients receiving donor SSCs generated functional spermatozoa and, in turn, viable offspring (Brinster and Avarbock 1994). In farm animals, using SSC transplantation, live offspring have been produced in goats (Honaramooz et al. 2003a) and sheep (Herrid et al. 2009a) with 7–10% efficiency. Since the generation of donor SSCs derived offspring is the

sole gold standard parameter that would affirm the success of SSC transplantation at the field level, enormous efforts have yet to be made to improve the efficiency of offspring production in livestock.

9.11 Conclusion and Future Prospective

Since its inception in livestock, SSC transplantation has shown tremendous potential for use in fertility restoration and as an efficient and cost-effective alternative approach for genetic modification. Despite the encouraging preliminary achievements, enormous efforts are yet to be made to improve the rate of live offspring production, and the proportion of donor SSCs derived spermatozoa in the recipient's semen. Future research in this area will mainly focus on developing reproducible protocols for the enrichment and long-term propagation of SSCs from livestock. There is a necessity to develop efficient and safer methods for SSC depletion in recipients, which will further enhance the colonization efficiency of the transplanted SSCs.

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Multiple Ovulation and Embryo Transfer in Livestock Production

10

Suresh Kumar Singla and Birbal Singh

Abstract

Conserving and propagating quality livestock germplasm is vital to enhance production and minimize the management cost of animals. Assisted reproduction techniques (ARTs) ranging from artificial insemination (AI) to advanced genome editing have been used to enhance production and value addition of the livestock products. Harnessing female reproduction potential is target of animal reproduction biotechnologists and animal breeders. Single ovulation embryo transfer (SOET) and multiple ovulation embryo transfer (MOET) are the methods to produce embryos to achieve more progenies from superior females. The processe(s) involve drug-induced superovulation that results in release of multiple oocytes from a donor as opposed to release of single or sometimes two oocytes under natural reproductive cycle. MOET has been used in many livestock species including cattle, buffaloes, equines, camels, pigs, sheep, and goats. This chapter summarizes a perspective on pursuit of MOET in domesticated large animals with emphasis on water buffaloes, the leading livestock contributing to high quality milk and meat in South-East Asia and Mediterranean countries.

Keywords

Embryo biotechnology · Livestock · MOET · Assisted reproduction

S. K. Singla (✉)
ICAR—National Dairy Research Institute, Karnal, Haryana, India

B. Singh
ICAR—Indian Veterinary Research Institute, Regional Station, Palampur, Himachal Pradesh, India

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10.1 Introduction

Animal agriculture is an integral element of human nutrition, health and livelihood, and national economy. Domesticated animals provide food (milk, meat, and eggs), fiber, manure, and mechanical power for agriculture operation, and serve as a source of income to farmers. Billions of people depend for livelihood of animals (Singh et al. 2009, 2019a). Cattle, buffaloes, pigs, and poultry occupy nearly 30% of the earth's terrestrial area (Steinfeld et al. 2006) and serve as worldwide assets with a commercial value of more than $1.4 trillion (Thornton 2010). In addition, less-studied livestock such as camels, equines, yak, mithun, and reindeer are domesticated in specialized agro-climatic niches of the planet (Singh et al. 2019a). Equines are primarily used as pack and gaming animals. Interest and demand for donkey or jenny milk have increased due to its nutritional and health (Altomonte 2019; Li et al. 2020) benefits and use in cosmetics (Kocic et al. 2020). Similarly, camel milk has multiple health benefits (Talarico et al. 2019; Wang et al. 2020).

Efficient strategies to increase herd size are central to success of entrepreneurism based on animals (Daly et al. 2020). Further, increasing consumer demand for animal-origin products and inability to enhance production of high biological value foods underscore the role of livestock to supply meat and dairy products. It is possible chiefly through use of advanced techniques to increase nutrient utilization and enhance reproduction efficiency of the livestock including, cattle, buffaloes, goats, sheep, and pigs.

10.2 Assisted Reproduction in Livestock Production

Scientific management of animal reproduction and breeding is crucial to livestock production. AI, in vitro embryo production (IVEP), sex pre-selection, and animal stem cell science are of immense concern in current concern. Utilizing and augmenting female reproduction potential are of top interest in commercial livestock management.

Embryo biotechnology makes efficient use of the oocytes producing ability of animals. Single ovulation embryo transfer (SOET) and multiple ovulation embryo transfer (MOET) are of paramount concern. MOET (Fig. 10.1) offers a splendid procedure to maximize the use of high-yielding animals. For instance, ovaries of a healthy cow can produce around 150,000 eggs during her lifespan, but under natural conditions, a typical cow produces only one calf per gestation. This implies that only one egg or oocyte is utilized to produce a calf. However, it is possible to stimulate the development and release of multiple oocytes simultaneously and release them from follicles. The released oocytes can be fertilized in vivo or in vitro to generate embryos to produce live offspring.

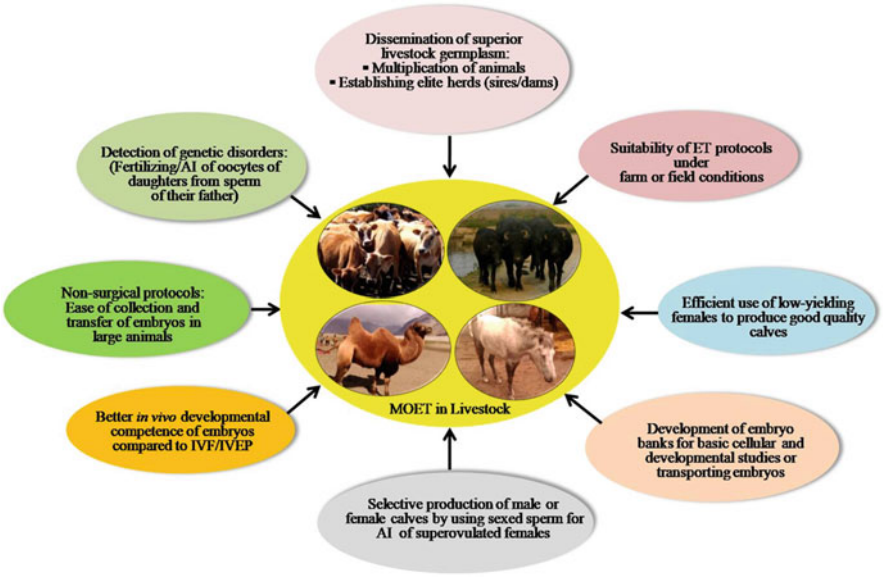

Fig. 10.1 Advantage of SOET and MOET to enhance population and productivity of livestock. The technique is used in most breeds of cattle, buffaloes, sheep, goats, pigs, equines, and camels

10.3 Purpose of MOET

Implementation of various forms of MOET, namely mature in vitro fertilization and embryo transfer (MIVET) and juvenile in vitro fertilization and embryo transfer (JIVET) in combination with genomic selection and AI or natural mating is the most efficient way to increase genetic gain, and thus increase the reproductive efficiency of animals (Daly et al. 2020).

The basic purpose is to disseminate genetically superior livestock though protocols that vary depending on species involved, requirements, and the objectives. MOET and JIVET can increase the number of offspring per mating or insemination, thereby increase the genetic gain (Granleese et al. 2015). JIVET reduces generation gap as oocytes can be harvested from animals before they reach maturity (Granleese et al. 2015). Box 10.1 summarizes various steps involved in a characteristic MOET protocol. Selection criteria (Table 10.1) should be followed stringently before proceeding to ET programs.

Box 10.1. Summary of Various Steps Followed in Typical MOET

1. **Selection of females:** Genetically superior females should be selected. Marker-assisted selection is recommended.
2. **Programming:** It involves intramuscular injection of FSH and prostaglandins.
3. **Detection of estrus:** Treated females are observed for onset of estrus. Care has to be taken in case of animals with silent estrus.
4. **Insemination of donor females:** Females are inseminated using fresh or frozen-thawed semen. Mating could also an option. Use of sexed sperm will yield embryos and offspring of desired sex.
5. **Collection of embryos:** In general, embryos are collected after 7 days of insemination or mating. Both uterine horns should be flushed for maximum recovery of embryos. Pregnancy should be terminated in superovulated females.
6. **Evaluation of embryos:** Embryos are evaluated under microscope for their quality. Various parameters are used to ascertain the quality of embryos. Healthy embryos can be spilt or bisect to develop two embryos from one embryo. This technique has been used in cattle and buffaloes.
7. **Preservation/transfer of embryos:** Freshly developed are transferred to estrus-synchronized females. Alternatively, embryos can be cryopreserved for commercial or basic and applied embryology and stem cells biology.

Table 10.1 A comparative overview of criteria to select potential donors and recipient females in embryo transfer programs

Donor females	Recipient
Genetic superiority to express quantitative traits regulating milk and meat quality. These traits are breed-specific	1. Reproductive and general health are most important criteria. Animals should be free from infectious diseases 2. Low-yielding healthy females are preferred as surrogate mothers
Animal should have normal reproductive physiology	1. Abnormalities in reproductive tract and physiology are not acceptable 2. Surrogates should have provided same diet for six weeks before estrus synchronization and ET
Donors should be in optimal condition body condition score for their breed	Donors should be in optimal health conditions.
Animals having any genetic disorders should be avoided	1. Animal should be synchronized to come into estrus 7 days before transplanting embryos 2. Females with recorded history of sound gestation and calving are the candidates of choice

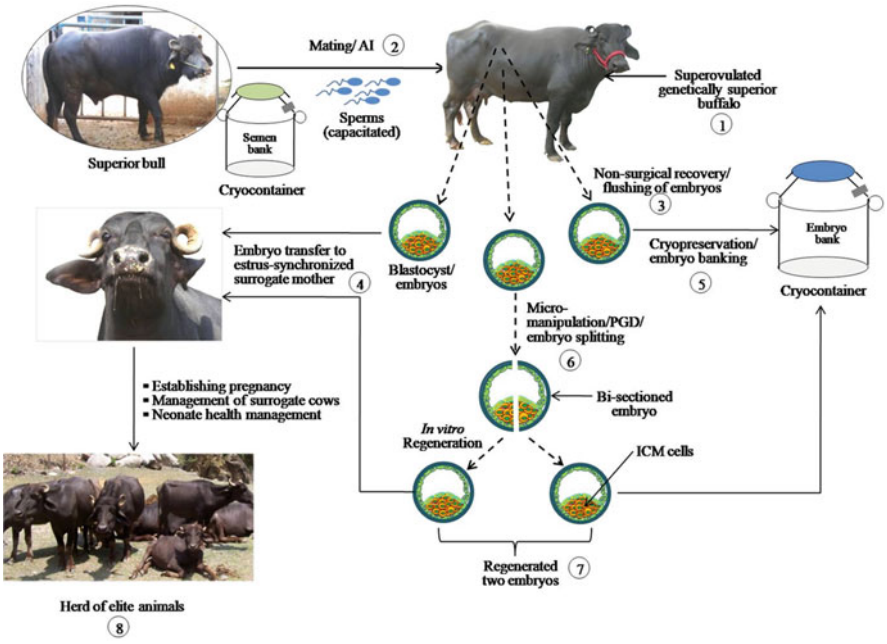

Fig. 10.2 Establishing herd of genetically superior livestock using MOET and associated embryo biotechniques. Inner cell mass (ICM) cells serve as sources of embryonic stem cells. The digits represent chronological steps used in a typical MOET process. (1) Selection and superovulation of donor female; (2) insemination or mating the superovulated with superior bull; (3) non-surgical recovery of embryos by flushing uterine horns; (4) ET to estrus-synchronized surrogate; (5) cryo-preservation of surplus or unused embryos, or embryo banking; (6) micromanipulation for generating isogenic embryos, or prenatal genetic diagnosis (PGD); (7) regenerated embryos from single bisected embryo; (8) an elite herd of buffaloes

MOET increases number of required male or female offspring at rate much higher that is possible through normal reproduction (Fig. 10.2). The MOET increases the reproductive capability and ability to produce valuable animals and simultaneously increase the genetic improvements of the herd (Rowe et al. 1980).

Outcomes of MOET are affected by several variables. Studies on various parameters affecting pregnancy rates in cattle have revealed that high quality fresh embryos transferred into surrogate females can achieve high pregnancy rates of up to 77.1% (Hasler 2001). MOET pregnancy rates were found to be comparable in dairy and beef cattle (Hasler 2001).

In addition, MOET is an effective means to prevent transmission of infectious diseases from herd to herd as well as geographically, or from dam to calf. MOET increases the reproductive capability and ability of precious livestock and increases genetic improvement within given time scale. When combined with advanced ARTs such as sperm sexing, it is possible to produce calves of desired sex to establish dairy and meat herds and produce chimera animals for developmental biological and

biomedical applications. Besides livestock, the ARTs have been used to conserve and repopulate the endangered wild and captive mammalian species (Mastromonaco and Gonzalez-Grajales 2020; Thongphakdee et al. 2020).

10.4 Drugs Used in SOET and MOET

Notably, MOET is an artificial mode of reproduction achieved by exogenous administration of drugs and biological(s). Follicle-stimulating hormones (FSH) are injected into selected females to stimulate recruitment and development of multiple ovarian follicles. In addition, it is necessary that females to be used as surrogate mothers should also be synchronized for onset of estrus so they cycle at correct time. Progesterone (e.g., CIDRs), PMSG (e.g., Pregnecol), GnRH (e.g., Receptal), and prostaglandin (e.g., Estrumate or Estroplan injection) are administered to donor females (https://www.abreeds.co.nz/moet; Accessed May 8, 2020). With the aid of FSH-superovulation a donor can produce 4–5 embryos at day 7 post-insemination or mating. The flushed embryos are evaluated for their quality. Embryos of acceptable quality are transferred directly to estrus-synchronized surrogates or preserved for later use or sale.

10.5 SOET and MOET in Various Animals

10.5.1 MOET in Cattle

Cattle are most important livestock species used for agriculture-based economies. Both *Bos indicus* and *Bos taurus* are used as multipurpose assets for milk, meat, skin, draught power, and manure. According to FAO, the world cattle inventory in 2016 has 1.474 billion head of cattle. Brazil has highest population of cattle, followed by India, China, and United States. Around 104 countries have cattle inventory in excess of one million head (https://www.drovers.com/article/world-cattle-inventory-ranking-countries-fao; accessed on August 20, 2018).

In addition to high-yielding recognized pure or synthetic cattle breeds, several native cattle found in many parts of the world that are known for their unique genetic and adaptive merits to survive and perform in difficult agro-climatic and low-input system. Indian bovine (*Bos indicus*) breeds such as Tharparkar, Gir, Kankrej, Mewati, etc. have climate resilient traits, thrive on highly fibrous forages, and perform well under heat stress. They are generally low-yielding animals and provide livelihood to marginal farmers. Ladakh cattle is evolutionarily adapted to low-oxygen high altitude milieu of Ladakh region (Verma et al. 2018). Hilly cattle of North West Himalayan Region (NWHR) are well adapted to grazing hilly terrains (Sharma et al. 2019). Characteristic dwarf Vechur cattle in Kerala are noted for its short stature, resistance to diseases and adaptation to hot humid climate (Sadan et al. 2020).

Use of superovulation combined with AI, and embryo transfer was an innovative method of multiplying desirable animals which commenced in cattle since early 1970s. Till 1990s, commercial embryo transfer started to be a widespread practice in commercial livestock farms. At present, OPU and IVEP are effective reproduction biotechniques in cattle (Mikkola et al. 2019).

Multiple ovulation and embryo development for cattle were initially developed in 1940s, but their large-scale applications were implemented after 1970s (Reviewed in Moore and Hasler 2017). Development of protocols for maturation of oocytes, sperm capacitation, fertilization, and embryo culture during 1970s and 1980s led to the birth of in vitro embryo developed calf in 1987 (Lu et al. 1987). In 1988, ovum pick-up (OPU) technique was developed to retrieve oocytes from live animals and develop the embryos by IVF (Pieterse et al. 1988). A rapid progress in in vitro embryo production (IVP) has been noticed since the 2000s with large-scale commercial applications (Reviewed in Moore and Hasler 2017).

Protocols developed in one species were applied to other species as well. Currently, MOET is widely implemented in cattle to increase the production of progeny, either males or females from genetically superior genotypes. Besides, MOET is used to test the inheritable genetic disorders of the bulls by mating or inseminating the bull with his superovulated daughters.

Several factors are responsible for success and outcome of superovulation. The number of oocytes that can be stimulated to grow and ovulate in donor are estimated by either ultrasound-guided sonography or by measuring the amount of anti-Müllerian hormone in the blood of females (Mikkola et al. 2019). Animal-related factors that can influence the efficacy of superovulation include animal breed, age, parity, genetics, lactational status, and reproductive history. In addition, nutrition, stress, season, climate, weather, and some semen factors also affect the success of MOET (Mikkola et al. 2019).

10.5.2 MOET in Camelids

The camelids are important livestock that play a role in their cradle of rearing.

The Camelidae family entails the Bactrian camel (*Camelus bactrianus*), the dromedary camel (*Camelus dromedarius*), and four species of South American camelids: llama (*Lama glama*), alpaca (*Lama pacos*) guanaco (*Lama guanicoe*), and vicuña (*Vicugna vicugna*) (Zarrin et al. 2020).

The interest for camels as valued adaptive livestock has increased during recent years. Camelids contribute to the economy of local populations by thriving on low-quality bushes, tree leaves, and forages, and have minimal water requirement. Camels produce valued milk, wool/fiber, and energy for transport and agriculture (Singh et al. 2019b; Zarrin et al. 2020).

Around 14 million camels with 90% dromedaries are present globally. Dromedaries are the domesticated camels mostly reared in Africa, The Sahel, Maghreb, Middle East and South Africa (https://en.wikipedia.org/wiki/Camel, accessed on Jan. 28, 2018).

The Bactrian camel is only truly wild (as opposed to feral) species found in Gobi and Taklamakan deserts of Mongolia and China. Somalia has largest populations of domestic camels, and Australia has largest number of feral camels (Singh et al. 2019b).

Over the past 3 decades, embryo production and transfer are practiced at commercial level in dromedary camel and alpaca (Herrid et al. 2017). Embryo preservation and transfer program were initiated in UAE since 1990s to meet the demands of camels for their multiple roles (Anouassi and Tibary 2013).

Notable, camels have characteristic reproductive behavior which differs from the livestock species. She camels are induced ovulators. They ovulate only when mated implying that ovulation should be induced before inseminating the females. Reports are available on IVF-derived embryos, cryopreservation of embryos, pregnancies, and birth of camel calves following ET as well as nuclear transfer cloning (Singh et al. 2019b).

Hormonal treatment is needed to induce ovulation and control follicular cycles which indicates that MOET and ET developed for other livestock species cannot be directly applied to camels (Vettical et al. 2016). A complete breeding soundness of donor and recipient or surrogate mother is required for ET programs in camels. Animals involved should be completely free from contagious diseases such as trypanosomiasis, brucellosis, and camel pox (reviewed in Vettical et al. 2016). Recipient females should be from 5–12 years old, in good general and reproductive health condition, and must have had one pregnancy and parturition (Anouassi and Tibary 2013).

Diagnosis and treatment of reproductive problems in donors before initiating superovulation are warranted. According to Anouassi and Tibary (2013), during 1990–2010, a total of 11,477 camel embryos were transferred into surrogates. Of 10,600 transfers during 1990–2009, a total of 2858 camel calves were borne which represent an overall efficiency (%weaned calves/embryo transfer) of 27%. It has been noted that finding good quality recipients is a major challenge (Anouassi and Tibary 2013). A combination of eCG and FSH gives best results for superstimulation of camel ovarian follicles (Skidmore et al. 2002; Wani and Skidmore 2010).

Embryos should be flushed on day 7 after ovulation and transferred to estrus-synchronized recipients on day 6 after ovulation. Notably, the recipients (having 13–17 mm ovarian follicles) are injected with GnRH or hCG after mating the donor (Vettical et al. 2016). The factors that affect embryo recovery include super stimulation treatments, reproductive and fertility status of donor as well as the male camel, embryo collection time after release and fertilization of ova, and the skill of personnel involved. Embryos can be recovered by surgical and well as non-surgical methods, but non-surgical recovery is preferred. The vitrification being simpler and advantageous should be used in camels (Herrid et al. 2016, 2017).

10.5.3 MOET in Equines

Humans have used equines in many ways for riding, pulling carts, travel, work, gaming, and shows. Cavalry was important component of war until middle twentieth century. Various breeds and species are domesticated in different home tracts.

Equine ARTs, viz. ET, MOET, embryo cryopreservation, oocytes from live or postpartum mares, intracytoplasmic sperm injection (ICSI), oocyte transfer, gamete intrafallopian transfer (GIFT), and nuclear transfer cloning have progressed swiftly during the past two decades (Roser and Meyers-Brown 2019; Singh et al. 2019c). Dearth of interest from equine breeders is one of the main reasons for tardy progress of ARTs in equines. In addition, poor response of mares to superovulation, complexities in stallion sperm capacitation, low IVF, and subsequent embryo development have impeded the embryo technologies.

Recombinant equine follicle-stimulating hormone (reFSH) and recombinant equine luteinizing hormone (reLH) have shown good results in mares (Roser and Meyers-Brown 2019). Variable results have been documented when eCG, GnRH, GnRH agonists, porcine FSH, domperidone, sulpiride, equine pituitary extracts, native equine FSH, human chorionic gonadotropin, progesterone, and immunization against inhibin were used to superovulate cyclic mares ((Roser and Meyers-Brown 2019).

Equine breeders have benefited from AI and IVF. The very fundamental purpose of using ARTs is to disseminate superior genotypes and treat infertility. The contemporary focus is to increase the success rates of ARTs for horse-rearing entrepreneurs and conserving the equine genetic diversity.

Athlete mares or those which suffer from orthopedic injuries or reproductive pathologies and unable to successfully carry or deliver a foal serve as potent donors. Unlike other livestock, such as cattle superovulation is poorly successful in mares. Hence, embryo recovery is poor.

hDG and GnRH are suitable to stimulate dominant ovarian follicles. The strategy is exclusively useful for aged mares (Riera et al. 2016). The mare is inseminated when follicles are about to rupture to release the ovum, or in coordination with induction of ovulation by administering GnRH or LH analogs (Hinrichs 2018). The embryo is flushed after day 7–8 of insemination. Various protocols are used to flush the embryos from uterine horns or uterus. The embryos are examined for quality and transferred or cryopreserved.

A single daily intramuscular injection of recombinant equine FSH in seasonally anovulatory mares exhibited multiple follicular development, release of fertile oocytes which on insemination developed to embryos that established pregnancies (Roser et al. 2020). The study shows that on average, reFSH administered for 6.5 days elicited ovulation in 80% of the mares treated. Follicles of size $\geq$35 mm, and 88% embryo rate per ovulated mare were achieved (Roser et al. 2020).

Prospective areas that need attention include improving oocyte maturation in vitro, sperm capacitation, IVF and proliferating the progeny-tested studs (Singh et al. 2019c).

10.5.4 MOET in Buffaloes

The buffaloes are multipurpose livestock species in South Asia, the Mediterranean Regions of Europe, and South America for over 500 years. Also regarded as "black gold," the buffaloes are important sources of milk, meat, manure, and draft power (Madan et al. 1996; Singh et al. 2009, 2020; Mann et al. 2013; Selokar et al. 2014, 2016). Most buffaloes are integral component of rural agriculture though can also be found in urban areas.

Among domestic buffaloes (*Bubalus bubalis*- Linnaeus, 1758), namely, the river buffalo ($2n = 50$), and swamp buffalo ($2n = 48$), the river or water buffalo exist predominantly in South-East Asia where nearly 97% of the global buffalo population including world's best buffaloes (e.g., Murrah, Nili Ravi, Surti, Jaffarabadi, etc.) are found. Many countries depend primarily on buffaloes for milk and meat besides other uses in agriculture (Gautam et al. 2008; Dev et al. 2012; Mann et al. 2013; Singh et al. 2020). Buffalo milk contains 7–8% fats and 4.2–5% proteins, which implies that milk has more contribution than its actual available quantity. Buffalo milk is highly suitable to make dairy products such as mozzarella cheese and butter oil. Compared to cattle, the buffaloes utilize efficiently fibrous roughage, grasses, tree foliage, crop residues, hay, and stover (Nanda and Nakao 2003) and perform better under tough climate of tropical regions.

In vitro embryo production (IVEP) and ET in buffaloes began first in USA with the first report of buffalo calf from ET (Drost et al. 1983). Thereafter, other countries also adopted the technology to produce embryos in vitro, for faster dissemination of buffaloes (Hufana-Duran et al. 2004; Madan and Prakash 2007).

Singla and Madan (1990) produced high quality embryos using SOET with 60% efficiency in non-superovulated buffaloes. A study conducted to record fertilization rate and embryo developmental rates in superovulated buffaloes showed that out of total 139 embryos, 27.4% were unfertilized, 17.9% degenerated, and 54.7% were of transferrable quality. Majority of the embryos reached blastocyst stage on day 6 of insemination. The study concludes that buffalo embryos have tendency to grow faster in reproductive tract (Singla et al. 1996). With improved superovulation protocols, the early recovery rate of 0.15 transferable quality embryos has already been increased to 2.0 (Madan et al. 1996).

MOET was initiated to expedite embryo production and increase the number of offspring from high-yielding buffaloes. Despite inferences that MOET is successful merely in organized scientific farms (Gandhi et al. 2007), MOET is practiced in several organized buffalo farms. It is noted that overall success rate in terms of calves born per superovulation is low for the MOET, hence prospects of the technique are low under field conditions (Perera 2008).

Gautam et al. (2008) have concluded in vitro production of embryos from abattoir-derived oocytes. Progress in embryo production may lead to faster propagation of valuable animals and boost the associated embryo-based technologies such as stem cells, genome-editing in vitro cell farming, etc. During recent years, success has been achieved to clone superior buffaloes (Selokar et al. 2014, 2019).

10.6 Prospects and Challenges of MOET

Despite prospects of ARTs, several challenges have to be resolved. Some ARTs are expensive and variations exist between ET recipients in their ability to sustain the pregnancy. Use of molecular markers for selection of donors and the recipients along with due management of recipient and the neonates are the essentially effective strategies. While ARTs enhance genetic gains by through selected genotypes, and lowering generation interval, some ARTs have serious limitations. Promotion of inbreeding is one of the most serious drawbacks that impedes the genetic diversity. Granleese et al. (2019) have suggested optimal contribution selection (OCS) method to be an effective method to keep the rate of inbreeding at a sustainable level without much effect on improvements in genetic gains. OCS could also be used to selectively and optimally allocate reproductive technologies in mate selection while accounting for their cost (Granleese et al. 2019).

Ironically, mechanization of transportation and agricultural activities has reduced the demand of male cattle and buffalo calves. Reproduction biotechnology has imperative role here. Sperm sexing should be improved to prepare doses of sexed sperm to produce female calves for establishing dairy herds and the males for meat. Prospective areas that need scientific attention include applications of MOET to conserve and proliferate the livestock which are evolutionarily adapted to low-input management under prevailing agroclimatic conditions, adverse topographic niche, poor-quality local forages and vegetation, and less requirement of water. Niche-specific livestock such as camelids should be proliferated to cater the area-specific needs.

Genome-editing technologies should be applied to decipher gene function and introduce beneficial gene variations into structured livestock breeding programs. This will produce livestock with superior adaptive and production traits.

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Ovum Pick-Up and In Vitro Embryo Production in Bovine

11

S. S. Layek, S. P. Patil, S. Gorani, K. Karuppanasamy, G. Kishore, and R. O. Gupta

Abstract

Ovum pick-up (OPU) and in vitro embryo production (IVEP) are envisaged to change the face of dairying across the globe. This biotechnique promises to multiply superior bovine germplasm with efficient use of sexed semen technology and inferring genomic selection indices at the embryonic stage to produce high genetic potential calves. Bovines well tolerate the technique, and its efficiency can be improved either by ovarian stimulation before OPU or by media modulation to provide a conducive growing environment for zygotes. Due to improved culture conditions, the conception rates are slowly getting at par with both fresh and frozen embryos. However, in future, integrating the work of genomics and artificial intelligence in OPU-IVEP would help fulfill the claimed promises generously. In this chapter, the process of OPU and in vitro embryo production are discussed in detail besides discussing the similarities and dissimilarities between in vivo and in vitro embryo production. Further, the futuristic views and potential of OPU-IVEP technology are also discussed.

Keywords

Ovum pick-up · In vitro fertilization · Bovine · Embryo · Assisted reproductive technique

S. S. Layek (✉) · S. Gorani · K. Karuppanasamy · G. Kishore · R. O. Gupta
National Dairy Development Board, Anand, Gujarat, India

S. P. Patil
College of Veterinary Sciences and Animal Husbandry, Kamdhenu University, Anand, Gujarat, India

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11.1 Introduction

There is a constant expansion of the commercial livestock industry to cope with the continuously growing demand of the human population. The vicious interplay between reproduction and production plays a critical role in effectual livestock farming. Unlike elite males, which can be propagated to tens or hundreds of thousands of offspring through artificial insemination, the contribution of a genetically superior female to breeding is limited by their monovulatory nature. Additionally, in dairy developed nations, the cows are usually culled before or around the third or fourth lactation liable upon various factors. Reproductive biotechnology, i.e., ovum pick-up (OPU) and in vitro embryo production (IVEP) along with embryo transfer (ET), pledge to redress the balance in favor of the superior females.

Louise Brown, the first human IVF child, was born in 1978, which created a ripple among the scientist to adapt and implement the technique to other large mammals, including the cow. Resulting, in 1982 the first live calf was born by transfer of an in vitro produced embryo (Brackett et al. 1982). IVEP was developed as a technique for bovines similar to that used for humans, i.e., treatment for infertility and basic research. However, later it becomes a technology to attain higher genetic gain. Through initial years for clinical application of IVF in infertile cows and mares, the preferred technique for oocyte recovery was either through laparoscopy, laparotomy, ovariectomy, or blind needle penetration via the paralumbar fossa (Sirard and Lambert 1986a, b). Until 1988, when oocytes from donor bovines were recovered using the ultrasound-guided transvaginal aspiration of ovarian follicles (Pieterse et al. 1988). The technique was adopted and modified for cattle and mares as it was developed for humans (Pieterse et al. 1991; Brück et al. 1992; Cook et al. 1992).

Protocols for in vitro embryo production (IVEP) were further developed in the 1990s, as an alternative to multiple ovulation and embryo transfer (MOET), by combining ovum pick-up (OPU), in vitro maturation (IVM), in vitro fertilization (IVF), in vitro culture (IVC), and embryo transfer (ET) (Looney et al. 1994). The Dutch team's discovery of the transvaginal ultrasound-guided aspiration technique proved to be a game changer. It has grown rapidly since the 2000s, with large-scale commercial operations established primarily in South America (Hasler 2014). The technique also remains the base for other biotechniques like cloning, transgenesis, and gene editing, all of which have, as a starting point, an oocyte or an embryo.

Across the world, in vivo embryo production is carried out through the MOET process. However, the technology requires costly hormones for superovulation and requires a significant resting period before using the same animal. During the last decade, OPU-IVEP has emerged as a replacement for in vivo embryo production technique. It is envisaged that the use of this technology for the multiplication of superior germplasm is paving the path of changing the face of dairying globally. In bovine, the use of OPU-IVEP of embryos by commercial has amplified worldwide. At present, OPU-IVEP embryos represent a sizable fraction of the total number of cattle embryos produced in the whole world surpassing the in vivo embryo production. The inception of genomic selection and sexed semen gave further impetus to

the technology. With genomic selection, an animal's genetic worth can be estimated at birth, and with OPU-IVEP, progenies can be obtained even before the female attains puberty. Further, the sexed semen can be most efficiently used using OPU-IVEP because oocytes from multiple donors can be fertilized using a single frozen semen dose making the use of it in the most cost-effective manner. The schematic representation of OPU-IVEP in bovines is given in Fig. 11.1.

The technology has got several benefits that facilitated faster adoption by several dairy-developed countries of the world. Following are a few very prominent benefits of the technology:

- The number of progenies from an elite female animal can be increased during its lifetime. By applying OPU-IVEP technologies, one can easily get 20–25 calves from an elite animal in a year compared to one calf normally obtained from an elite female animal in 1-1.5 year through artificial insemination.
- It helps increase the selection intensity, which means that we can produce more calves from selected smaller number of top elite animals using this technology. Thereby increasing genetic gain per year and speeding up the rate of genetic improvement.
- Further, pre-pubertal elite heifers nominated by genomic selection can be enrolled early to produce calves, which would reduce the generation interval and thereby increase genetic gain per year.
- Further, oocytes can be collected from pregnant (during the first trimester) and juvenile donors, which facilitate shortening of generation interval and thus contributes to genetic improvement.
- Exceptionally high producing female animals with kink cervix and similar reproductive problems could also be used to take progenies.
- Several superior bulls can be used to fertilize oocyte pools in the same OPU-IVEP cycle to produce calves from different sire dam combinations in a very short period.
- Use of sexed semen can further improve the gain by producing offspring of desired sex in higher numbers.
- Science is developing faster than ever, and more benefits may be available for the bovine industry, such as selection of sex of the embryo before transferring, estimating genomic breeding value through embryo biopsy, detecting genetic diseases in the pre-implantation embryos, etc. These would further enrich the value of the OPU-IVEP technique.

The present chapter will focus on different facets of this promising technology to develop an understanding of this highly promising technology about the application, challenges, and future scope of the technology.

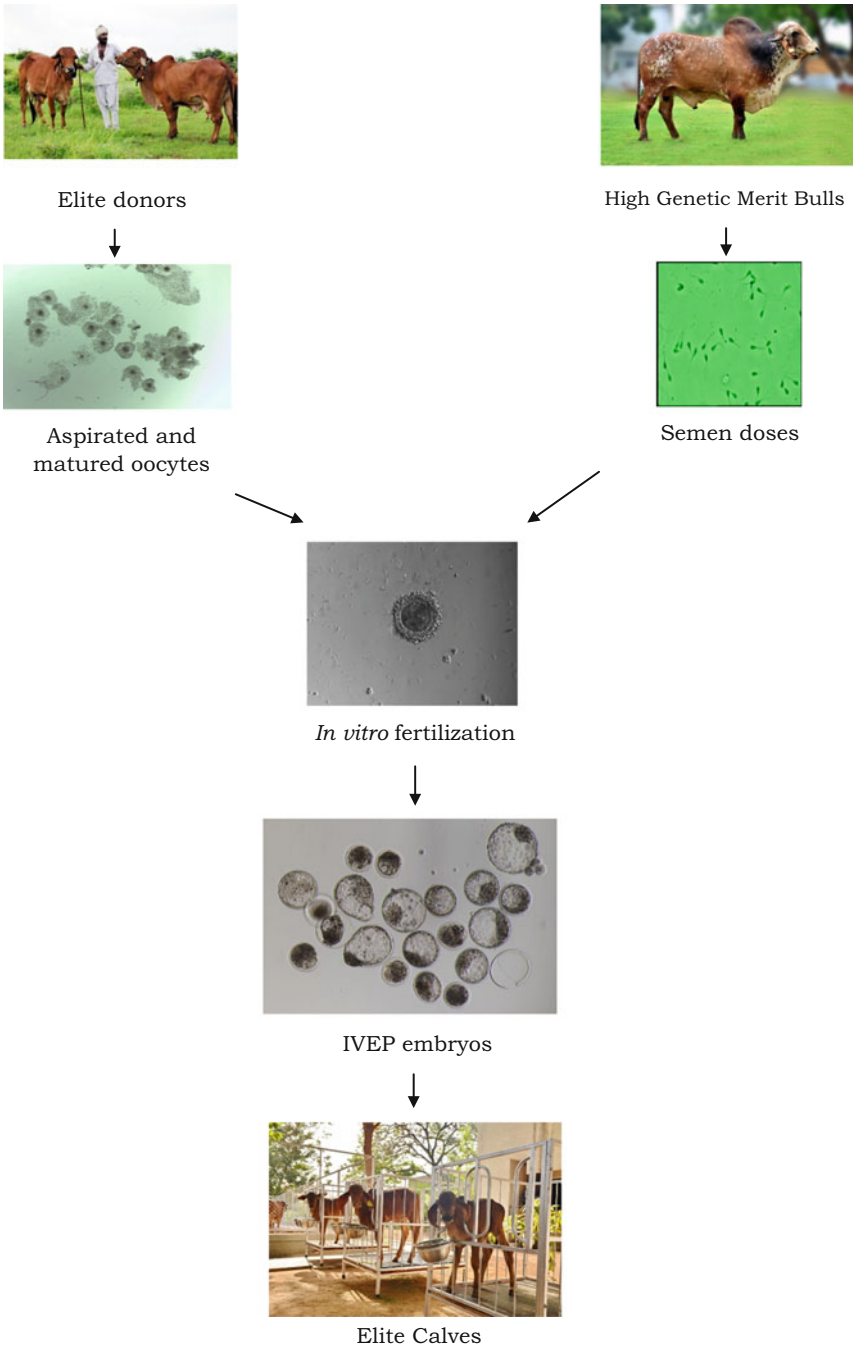

Fig. 11.1 Schematic representation of OPU-IVEP in bovines

11.2 In Vivo Versus In Vitro Embryo Production Systems

In vivo (Latin for "within the living") generally refers to the technique of performing a given procedure (in this case, fertilization or formation of zygote/embryo) within a living organism, which implies the production of embryos inside the fallopian tube/uterus of animals. In vitro (Latin for "within the glass") refers to the procedures performed in a controlled environment outside of a living organism, implies the production of embryos in a Petri dish, this in human being popularly known as "test-tube baby."

In vivo embryo production is carried out through multiple ovulation and embryo transfer (MOET). The application of MOET technology requires, in bovine (mono ovulatory) species, administration of hormones (Follicle Stimulating Hormone—FSH) to produce multiple ovulatory follicles and subsequent multiple ovulation (also referred to as superovulation). Such superovulated elite females (the donors) are either artificially inseminated or naturally mated, and their uterus is flushed to recover embryos 7 days post insemination. Embryos thus produced are evaluated under the microscope, and good quality embryos are either transferred to synchronized recipients or cryopreserved for subsequent transfer to a synchronized recipient in the future.

Whereas IVEP is carried out through the collection of oocytes obtained either from abattoir-derived fresh ovaries or from live animals through the OPU technique guided by ultrasonography (USG). Oocytes obtained through both techniques are evaluated under the microscope. Good quality oocytes are selected and matured and fertilized using fresh/ frozen-thawed semen in petri dishes in the controlled environment inside a specialized incubator for in vitro embryo production. Embryos are grown by culturing in specialized media up to the morula/blastocyst stages within a week. The embryos could be transferred or cryopreserved at this stage only for better conception. The OPU technique is considered more consistent than MOET. It permits safe and repeatable embryo production without using exogenous hormones and affecting normal milk production and altering the reproductive cycle.

Both in vitro and in vivo embryos are cryopreserved and transferred in the same manner. So, the difference lies in the production method of both types of embryos, and there is no difference in how they are transferred and cryopreserved. In bovines, the use of IVEP by commercial embryo production companies has increased. There are multiple similarities and differences between in vitro and in vivo produced embryos (Greve et al. 1992; Leibo and Loskutoff 1993; Avery and Greve 1995; Van Soom et al. 1996). The differences are the obvious outcome of the artificial culture condition and thus require a closure look, particularly in the in vitro culture system post-fertilization (Lonergan et al. 2003; Blondin 2017). The similarities and dissimilarities between in vivo and in vitro embryo production systems are given in Table 11.1, and the comparison between in vivo and in vitro produced embryos in terms of the production system is given in Table 11.2.

Table 11.1 Similarities and dissimilarities between in vivo and in vitro embryo production

Similarities	• Diameter at different stages of growth is similar between embryos produced through in vitro and in vivo methods. • Sperm penetration rate is comparable between the procedures. • There is no difference in fertilization and cleavage rates, and the time for cleavage is also similar between the methods. • There is no difference in the number of total embryos, the number of embryos having more than 16 cells, the number of transferable embryos, and the number of cells in the transferable embryos. • There is no difference in their transfer techniques.
Differences	• Morphologically, in vitro embryos are darker, less compact, have a comparable lower cell number, have more fragile zona pellucida, have a low ratio between inner cell mass and trophectoderm. • In vitro produced embryos are more fragile to cryo shock and have comparatively reduced viability following freezing. • Nuclear maturation is found to be more rapid in the case of in vitro embryos • Pregnancy rate is 10–15% lower in the case of in vitro produced embryos when compared with pregnancy rate obtained through the transfer of in vivo embryos. • However, the cost of producing in vitro embryos is much less than in vivo embryos. Hence, in vitro embryos are available at much cheaper rates. Nowadays, their availability in the international market is comparatively more. • There is occasional birth of a seriously overweight calf or calf with congenital anomalies when in vitro embryos are used. • Prior investigations detailed that various microorganisms are bound to remain related with in vitro derived embryos. However, with modified protocol and biosecurity measures, the risks of disease transmission are reduced to negligible proportions.

11.3 Understanding the Technology: Critical Look Through the Steps

The ultimate aim of IVEP is the same as that of MOET, i.e., production embryos for transfer but the method employed are very different. Though the technique in human is commonly known as IVF, it has a few distinctly divided steps, i.e., ovum pick-up, in vitro maturation, in vitro fertilization, and in vitro culture (Looney et al. 1994). Several established and emerging biotechnologies, including somatic cell nuclear transfer, gene editing, and production of stem cells, also demand proficiency in IVEP procedures as IVEP forms the base for these techniques.

Although the IVF technique has been mainstreamed in the past two decades, the advances are accelerating with no signs of slowing down. The inventive experimental efforts date to 1950 in the rabbit (Chang 1951) and rat (Austin 1951). The rabbit was the first mammal to deliver live offspring produced by IVF (Chang 1959), and subsequently, progenies to hamsters and mice were also produced through the IVF technique (Iwamatsu and Chang 1969). However, IVF protocols developed for rabbits and mice were initially unsuccessful for cattle. Surprisingly, an inadvertent finding of the capacitation-inducing ability of heparin on bovine spermatozoa in vitro fast-tracked the success of IVEP (Sirard 2018). In 1981, first calf was born

Table 11.2 Comparison between in vivo and in vitro produced embryos in terms of production system

Parameters	In vivo embryo production	In vitro embryo production
Type of donors	Only cyclic heifers and cows	Cyclic and non-cyclic heifers and cows, juvenile heifers, early pregnant (first trimester), donors with blocked oviducts, old, crippled donors
Interval between procedures	Four to 6 weeks	Two weeks (every week if un-stimulated)
Total possible procedures/ year	Up to 6–8	Up to 20–25
Av. viable embryos/ procedure	Up to 6	Up to 5
Use of sex-sorted semen to produce embryos of desired sex	Very limited, due to low efficiency	Strongly recommended, due to very high efficiency
Total embryos/donor/year	35–50	100–125
Pregnancy rates with fresh embryos	60–70%	45–50%
Pregnancy rates with frozen embryos	50–60%	35–45%
Total calves/donor/year	21 to 35	45 to 63
Cost/female calf produced	High	Lower

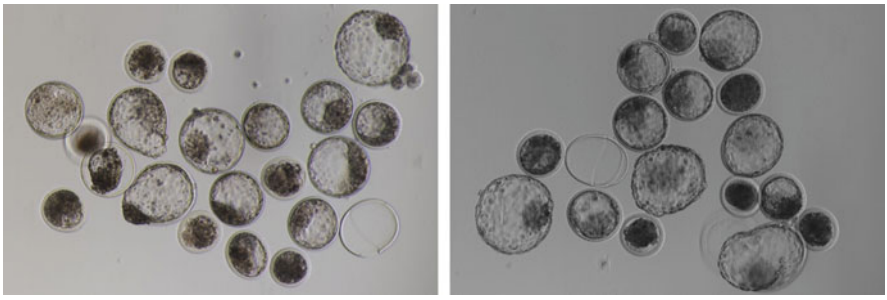

Fig. 11.2 Bovines embryos produced through OPU-IVEP Method

from IVF of in vivo matured oocyte (Brackett et al. 1982). Further refinement of IVEP steps finally led to the birth of the first calf following all IVEP procedures (Lu 1987). The developments happening over time continuously kept triggering for establishing an optimal culture condition for each step of IVEP.

In vitro development is assessed at three stages of the process, first following IVM, based on the expansion of the cumulus cell layers, second at around 48–72 h post gamete coincubation, when they are assessed for fertilization and development based on the cleavage to two or more cells and finally on Day 7 when the proportion of inseminated oocytes is assessed for development to the blastocyst stage (Fig. 11.2). The process has evolved through many changes, and presently, a pretty

Fig. 11.3 OPU in cattle and buffaloes in farm and field condition

stable protocol is in vogue, enabling the production of embryos on an industrial scale. However, the requirement for further improvement is always there, particularly in the area of in vitro maturation, blastocyst rate, and cryo-tolerance (Lonergan and Fair 2016).

(a) Ovum Pick-up

Transvaginal ultrasound-guided oocyte aspiration (TVOR), transvaginal follicular aspiration (TVA), or ovum pick-up (OPU) is a swift and minimally invasive technique for retrieving oocytes from a donor female repeatedly. OPU can be performed with or without prior exogenous hormonal stimulation. Performed under epidural anesthesia (occasionally combined with mild sedation), the OPU technique is harmless, repeatable, and more consistent than MOET. It allows safe and repeatable embryo production without affecting the normal milk production or altering the reproductive cycle.

The basics of the method involve per rectal manipulation of an individual ovary against a transducer (5–7.5 MHz) fixed inside a needle guide. This needle guide assembly is then inserted per vagina of a donor cow or buffalo (Fig. 11.3). Oocytes from all visible follicles (2–8 mm) are aspirated irrespective of the stage of follicular growth. Both dominant and subordinate growing follicles from

follicular waves are aspirated (Hyttel et al. 1997). The visible follicle (≥3 mm) is aligned against the puncture line on the USG monitor, and a needle (18–20 G) is inserted to aspirate the follicular content under vacuum pressure. Once detached from the follicular wall due to negative pressure, the oocyte and surrounding cumulus cells [cumulus oocyte complex (COC)] are aspirated into the aspiration line to the collection tube containing OPU media.

(b) Oocyte Recovery and Aspiration Frequency for OPU

Oocyte recovery rate, i.e., oocytes collected per follicle aspirated, varies with species, individual animals, follicular maturity, stage/day of the estrous cycle, needle size, vacuum pressure, and technician experience performing the aspiration (Merton et al. 2003). Altering needle gauge and/or vacuum pressure may improve oocyte recovery rates. However, higher pressure tends to strip off the cumulus cells, increasing the proportion of denuded oocytes (Bols et al. 1995; Fry et al. 1997). The quality of an oocyte is primarily judged based on the number of cumulus cell layers surrounding an oocyte (Viana et al. 2004). Needle size and vacuum pressure are to be optimized. Maximum oocytes are aspirated under good quality grade because fertilization rates and subsequent developmental competence reduce for lower grade oocytes (Tanghe et al. 2002). However, in contrast, it has been observed that despite the lower grade of OPU derived oocytes, these oocytes have higher developmental competence than oocytes derived from slaughtered ovaries. Therefore, there are high chances that lower grade oocytes recovered through OPU may convert into good quality blastocysts. Therefore, oocytes falling under lower grades, i.e., Grade III and Grade IV, are considered culturable and can be subjected to IVEP protocols (Neglia et al. 2003).

The frequency of OPU sessions influences both the quality and quantity of oocytes (Merton et al. 2003). The frequency of oocyte aspiration in donors (open, pregnant, pre-pubertal) varies from once/twice a week to once in fortnight interval with or without stimulation (Chaubal et al. 2007). In cattle, twice-weekly follicular aspirations, i.e., at 3- to 4-days interval, prevents selection of a dominant follicle; therefore, this allows for frequent aspirations and tends to be effective in harvesting more COCs from each aspiration session (Kruip et al. 1994; Broadbent et al. 1997; Garcia and Salaheddine 1998; Merton et al. 2003).

(c) *Bovine's Response to OPU*

OPU-IVEP technology is highly developed in South America (primarily Brazil) and has been implemented in *Bos indicus*, *Bos taurus*, and *Bos indicus-taurus* breeds of cattle. The primary difference in oocyte recovery among the latter states is that there is a larger population of small follicles and a higher number of follicles recruited per wave in *Bos indicus* and *Bos indicus-taurus* breeds compared to *Bos taurus* breeds (Viana et al. 2000). Therefore, *Bos indicus* and *Bos indicus*-taurus breeds yield a higher number of oocytes during OPU under normal conditions than *Bos taurus* (Baruselli et al. 2012; Watanabe et al. 2017). For instance, a study on IVEP from dairy Bos taurus, *Bos indicus*, and indicus-taurus donors was done at commercial dairy herd units. The data reported that the number of viable oocytes per OPU was 12.1 ± 3.9 for Gir cows, 8.0 ± 2.7

for Holstein cows, 16.8 ± 5.0 for 1/4 Holstein x 3/4 Gir, and 24.3 ± 4.7 for 1/2 Holstein-Gir crossbred females (Pontes et al. 2010). Similarly, beef *Bos indicus* breeds like Nellore, on an average per OPU, yielded 30.8 ± 0.9 oocytes (Pontes et al. 2011). Furthermore, there is a great degree of variation within the animals of the same species, breeds, and age, in the collection of oocytes. However, there is not much difference in the oocyte recovery in the same animal between OPU sessions (Bols et al. 2005). Therefore, it is said, "*A good donor is always a good donor*," which holds for OPU-IVEP and MOET.

(d) *Strategies to improve OPU efficiency in reference to IVEP*

Typically, of all in vitro matured and in vitro fertilized oocytes, only about 20–40% of oocytes develop to form transferable embryos. The majority of cultured oocytes/presumptive zygotes arrest/cease development at some point in IVEP (Wrenzycki 2016). One of the possible reasons is, on the day of OPU, the follicles present on the ovary are in varying stages of growth, e.g., oocytes in large follicles are undergoing a process of prematuration essential for attaining developmental competence for fertilization and further embryonic development (Hyttel et al. 1997). However, the same may not be true for oocytes present in smaller follicles, which may still be in growing stages or initiate atretic changes. Such oocytes do not usually translate into embryo development. Therefore, to ensure a homogenous follicular pool and modify oocyte quality, follicular dynamics of the donor is manipulated to synchronize follicle wave emergence and later is stimulated with exogenous gonadotropin-like Follicle Stimulating Hormone (FSH) before OPU to obtain a maximum pool of homogenous follicle for aspiration (reviewed by Seneda et al. 2020).

Follicular wave synchronization and stimulation before OPU promised boosted results for IVEP and pregnancy rates in bovines (Cavalieri et al. 2018). Therefore, under commercial livestock IVEP facility, it is the most common strategy used to increase COC recovery and enhance embryo production. A recent report, analyzing 12 years of practical data of a commercial dairy farm, stated real-world strategies to improve OPU-IVEP using synchronized and stimulation of donors before OPU (Demetrio et al. 2020). Stimulated OPU renders technicians aspirate more follicles, thereby increasing oocyte recovery translating into more embryos than non-stimulated OPU. The basic wave synchronization and stimulation regimen include the following (Blondin et al. 2002):

(i) Induction of follicular wave emergence to initiate stimulation.
(ii) Stimulation of the targeted/induced follicular wave with exogenous gonadotropin.
(iii) Allowing adequate coasting duration before OPU for achieving oocyte developmental competence.

Wave emergence and synchronization can be achieved before OPU hormonally or mechanically. The most preferred hormonal approach consists combined use of estradiol and progesterone. Considering the ban on estradiol-17β, a combination of estrogen esters (estradiol benzoate) administration along with implantation of a progestin device to negate the

estrogen-influenced LH release is used. The combination of the latter induces wave emergence 3–4 days later, unrelated to the stage of follicular growth at the time of treatment (Bó et al. 2002). Dominant Follicle Removal/ablation (DFR) is a mechanical approach involving transvaginal ultrasound-guided ablation of dominant follicles ≥5 mm, regardless of the stage of the estrous cycle (Bergfelt et al. 1994; Lima et al. 2007). DFR eliminates the suppressive effect of estradiol and inhibin from the dominant follicle on FSH release. Therefore, this causes an FSH surge inducing a new wave emergence a day later of DFR (Adams et al. 1992). Although DFR is difficult to utilize under field conditions, considering a ban on estrogenic compounds, it is the most effective tactic to induce and synchronize follicular wave emergence.

Post-inducing or synchronizing wave emergence, the targeted follicular wave is stimulated using exogenous gonadotropin-like FSH. The preferred stimulatory regime comprises four or six intramuscular administrations of FSH given 12 h apart over 2 or 3 days. The dose of FSH required per stimulation is usually half (200–300 mg) of that used in MOET, followed by a "coasting" period (FSH starvation) of roughly 40-50 h before aspirations. A wide range of studies evaluated the quality and quantity of COCs recovered post-wave synchronization and stimulation before OPU (Ongaratto et al. 2015; Baruselli et al. 2016). These studies concluded the following: (1) Follicular wave synchronization before OPU increased oocyte recovery and embryo production in Bos taurus, but not in *Bos indicus* breeds; (2) Dominant follicle ablation or combined use of estradiol and P4 had a similar response to induce and synchronization of follicular wave emergence for OPU; (3) Follicular stimulation with FSH improved the quantity and quality of oocytes aspirated from *Bos taurus* breeds (Bó et al. 2019). Stimulated OPU-IVEP boosted the rate of embryo production in Holstein donors (Vieira et al. 2014). Nevertheless, to counter multiple FSH administration and reduce animal handling, FSH is diluted in 0.5% hyaluronan. Total FSH dose is administered as single im administration, which is equally efficacious (Vieira et al. 2015). In *Bos indicus* cattle, FSH stimulation boosted the response to OPU-IVEP (Fernandes et al. 2014; Cavalieri et al. 2018); however, some studies have reported undesired results debating the use of FSH in *Bos indicus* breeds (Monteiro et al. 2010). Regardless, as stated above, *Bos indicus* breeds having more follicular population yield more oocytes through OPU; therefore, stimulation is non-essential. Farmers prefer this and do not synchronize and stimulate *Bos indicus* donors due to cost-effective outcomes.

(e) *Laparoscopic ovum pick-up (LOPU)*

Ongoing efforts to propagate superior bovine germplasm have led to newer generations of elite donor calves. There is strong driving interest in South American nations to accelerate the dissemination of these superior genetics. Therefore, revived by (Baldassarre et al. 2018) laparoscopic ovum pick-up (LOPU) technique, primarily designed for small ruminants (Cognié et al.

2003), is gaining popularity in the bovine side of OPU-IVEP. This is also known as juvenile IVF and embryo transfer (JIVET). The ovaries of juvenile animals have a higher population of antral follicles so that generally, more oocytes are recovered from young animals per OPU session (Landry et al. 2016). Nevertheless, the developmental competence of these oocytes to produce viable embryos is lower compared to oocytes of cyclic heifer or adult females (see Baruselli et al. 2016). Moreover, fewer studies have reported increased IVEP through calves when pre-treated with gonadotropin-like recombinant FSH (Taneja et al. 2000).

(f) *Long-term effects of OPU on donors*

Although bovines well tolerate the technique, there can be occasional incidences of intrafollicular hemorrhages (Chastant-Maillard et al. 2003). There is a prolongation of the luteal phase in bovines when pre-ovulatory follicles are luteinized post-aspiration. Such luteinized ovulatory and subordinate follicles tend to have a shorter life span and produce a lower concentration of progesterone (Petyim et al. 2000) but cyclicity and fertility of donors remain uncompromised (Kruip et al. 1994). Intensive use of donors for a prolonged period can cause a decrease in the number of follicles available, and thus oocyte yield is reduced. Although there are benefits of using exogenous hormones, extended use of the same can disrupt normal hormonal physiology and compromise the future fertility of the donor dam. Therefore, hormones should be ideally used for a shorter period allowing proper rest between frequent stimulation so that the natural cycle can regulate and resume normalcy (Qi et al. 2013).

Finally, as the OPU session comprises at least one epidural anesthesia, repetitive epidural or puncture at improper sight can result in the proliferation of connective tissue in and around the epidural canal (McEvoy et al. 2006). Even though not life-threatening to dams, the incidence should be reduced either by altering the needle size or injection site.

(g) *In vitro maturation (IVM) of oocytes*

The ability of bovine oocytes to spontaneously resume meiosis in vitro once liberated from follicles (Pincus and Enzmann 1935) is exploited greatly in IVM for IVEP. To one's surprise, maturation media preferred almost 30 years ago continue to remain the same till now. TCM-199 enriched with gonadotropins (FSH, LH), bovine serum albumin as a protein source along with growth factors like epidermal growth factor (EGF) constitutes to form IVM medium (Sirard et al. 1988). Similarly, the Tyrode-Lactate medium has been continually used for sperm washing and sperm/egg coincubation for many years. However, culture conditions for the final development of presumptive zygotes have been modified several times in the initial years and are still evolving. Most media for IVF, containing serum, are based on a balanced salt solution, amino acid solutions, and pyruvate. Vitamins, EDTA, and metal ion buffers are supplemented further.

There is an excellent maturation rate of oocytes subjected to IVM, but the number of oocytes developing to blastocyst is still around 40%. Therefore, efforts are being carried out to revisit the first step of IVEP to increase the

development competence of oocytes. In vivo, oocyte growth, and development are a tightly orchestrated process characterized by major nuclear and cytoplasmic events. As the oocyte approaches the final differentiation steps and during maturation, transcription is silenced. The regulated translation of stored mRNAs takes over to control gene expression until the embryonic genome is activated (Graf et al. 2014). The oocyte being the most susceptible to physiological and environmental factors (Hansen et al. 2016), the quality of an oocyte can be altered at any point of improper IVM conditions affecting the overall IVEP success.

The developmental competence of an oocyte is capped the moment it is removed from the follicle (Lonergan and Fair 2008). Few approaches are employed to obtain the most developmentally competent oocyte from the follicle itself. FSH coasting is one approach to address the oocyte quality. Terminal step of stimulation regime before OPU, i.e., withdrawing FSH (coasting) after several days of stimulation. The premise of the coasting strategy is that it mimics natural phenomena occurring before ovulation (Blondin et al. 2002). Oocyte quality is highest when coasting of 48 h. is observed, and follicular differentiation has reached the optimal stage under the influence of basal LH environment before harvesting oocytes. The quality and competence of the oocytes retrieved for IVEP are excellent, resulting in blastocyst development rates of up to 80% (Blondin et al. 2002).

Prematuration of the oocyte in vitro is another approach to enhance oocyte quality. Through OPU, oocytes are obtained from a heterogeneous pool of follicles days before ovulation. Oocyte collected during the follicular growth phase exhibits very compact and bright cumulus and is incompetent to develop. Early cumulus expansion can be correlated with the plateau phase and yield better results but not closer to 100%. Partially denuded oocytes are probably undergoing atretic and have very poor developmental competence compared to oocytes in the plateau group (Sirard 2011). Pre-ovulatory follicles yield oocytes with a maximum in vitro developmental competence (Dieleman et al. 2002). Although oocytes showcase high nuclear maturation rates, the time required for cytoplasmic maturation is longer. Therefore, IVM protocol to include a prematuration treatment by adding pharmacological inhibitors to reversibly inhibit meiotic resumption oocytes would provide the oocytes with the opportunity to undergo cytoplasmic maturation without resuming meiosis (reviewed in Bilodeau-Goeseels 2012).

(h) *In vitro Fertilization (IVF) of matured oocytes*

After maturation, oocytes are co-incubated with motile sperms for up to 18–24 hours to achieve in vitro fertilization. The gamete interaction occurs in a microenvironment of 50–100 µl for OPU oocytes (Gordon 2003). The frozen-thawed spermatozoa of chosen sire must undergo a series of steps to achieve fertilization potential. As occurring in vivo, while the sperm ascend the female reproductive tract, they undergo a series of biochemical changes, first independently recognized by Austin and Chang as the phenomenon of sperm capacitation (Austin 1951; Chang 1951). Capacitation destabilizes the sperm membrane,

allowing spermatozoa to bind to the oocyte zona pellucida (ZP) (Parrish et al. 1986; Parrish 2014). Sperm binding the ZP initiates the acrosomal reaction, enabling the sperm to penetrate and fertilize the egg (Breitbart et al. 2005). Sperm capacitation and the acrosome reaction must be recapitulated in vitro for successful IVF. The spermatozoa are washed and subjected to swim-up or density gradient centrifugation procedures in the laboratory. This allows selecting the more motile sperm fraction and removing freezing media, seminal plasma, debris, and dead spermatozoa. The spermatozoa are then exposed to capacitating agents like heparin, serum albumin, epinephrine, penicillamine, hypotaurine, caffeine, bicarbonate, and calcium to induce capacitation enabling sperms to penetrate the zona of the oocyte (Parrish et al. 1986). The concentration of sperms required per oocyte varies largely between bulls and breeds. However, most laboratory protocols inseminate with one to two million spermatozoa/ml for IVF (Ward et al. 2002). Most IVF protocols entail co-culturing sperm with oocytes enclosed with cumulus, as oocyte cumulus cell secretions such as P4 and hyaluronic acid trigger the sperm acrosome reaction. Compared to denuded oocytes, oocytes enclosed by cumulus had a higher proportion of live acrosome-reacted spermatozoa, with higher fertilization, cleavage, and blastocyst development (Fukui 1990). The fertilization rate is measured at 48-72 h post insemination and termed as cleavage rate. The cleavage rate usually ranges between 70% and 85%. Intracytoplasmic sperm injection (ICSI) is a well-used technique in human IVF but is still far from successful in bovines. Unlike human, the primary reason is that there is no spontaneous activation of oocytes after sperm injection. Other reasons include the darkness of the ooplasm, the large sperm heads, and the toughness of the oolemma (Abdalla et al. 2009).

(i) *In vitro culture (IVC) of presumptive zygotes*

Presumptive zygotes are transferred to IVC approximately 18-24 h post insemination, where they remain for up to 7 days until they reach the blastocyst stage. This embryo development stage is the most suitable for transfer into recipients or for cryopreservation for future timed transfer. Throughout the days of in vitro culture, the developing embryo undergoes several major events like switching the maternal genome to activation of the embryonic genome.

In wide-ranging IVF set-ups, only 20% to 40% of fertilized oocyte develops to the transferable stage (Rizos et al. 2008). Suboptimal results post in vitro culture were a challenge for scientists to develop an ideal culture environment for growing embryos. Dynamic conditions in vivo (oviductal fluid) nourish and fulfill an embryo's different metabolic requirements at early stages. Therefore, earlier scientists cultured bovine embryos post-IVF within oviducts of rabbits (Sirard and Lambert 1986a, 1986b) and sheep (Lu et al. 1987). In due course, coincubation with oviductal cells took over oviductal culture, which was believed to be essential to surpassing the 8-cell block in bovine embryos (Eyestone and First 1989). Adequate knowledge of the oviductal environment led to the preparation of Synthetic Oviduct Fluid (SOF) (Tervit et al. 1972). SOF

with few additional supplements is used as a continuous culture medium system for in vitro culturing developing embryos (Holm et al. 1999).

The metabolic requirement of an embryo varies at every developmental stage. Following that theory, a sequential culture system was developed and adopted widely in human IVF labs. Nevertheless, the culture system is shifting back towards a monoculture system supplemented with all required nutrients allowing the developing embryo to choose the nutrient required at its particular stage of development (Gardner et al. 2002). The prime advantage of a monoculture system is that the embryos are manipulated at least to none until day 7, lowering the chances of fluctuations, particularly of pH and temperature, which are said to be the worst enemy of developing embryos (Swain 2010). Additionally, the presence of serum in culture media has improved developmental rates to the blastocyst stage (Eyestone and First 1989). Still, then again, few reports suggest an association of serum in culture media with aberrant embryo morphology, poor cryo-tolerance, altered gene expression (Rizos et al. 2002), and, more recently, inappropriate DNA methylation (O'Doherty et al. 2018). In the late 1980s, serum-based culture media was also the culprit for the large offspring syndrome (LOS) appearance. LOS is a phenomenon of epigenetic origin that leads to dystocia due to large or malformed calves (Lazzari et al. 2002). The occurrence of LOS left us with a lesson that minor media modulation may result in noticeable undesired effects in produced calves. At present, bovine serum albumin (BSA) took over to serum to formulate serum-free media and is working at par and often even better blastocyst rate than serum-based media (Stroebech et al. 2015). Replacing serum with BSA also favored worldwide import/export of embryos as serum can be a potent carrier of pathogens. Over the years, considering the developments for laboratory instruments like the type of incubators and scientific developments in media modulations by augmenting various growth factors, antioxidants, etc., the average rate of embryo production seldom crosses over 30–40%.

Since the inception of bovine IVF, it was apparent that IVEP embryos did not resonate similarly to their in vivo counterparts. Typically, when compared between an in vivo derived blastocyst, and an in vitro produced blastocyst, the latter differ significantly at metabolic profiles, ultrastructural character, and at the genetic level compared to IVD blastocyst (Hansen 2020). Moreover, the implantation rate and ability to survive cryopreservation are also less in IVEP embryos than IVD embryos (Pryor et al. 2011; Alberto et al. 2013). However, when the culture system is reversed, i.e., when IVEP bovine zygote is subjected to in vivo systems (ewe or cow oviduct), it produces blastocyst similar to IVD blastocyst. On the other hand, IVD bovine zygote produced a very poor-quality embryo when cultured under a laboratory system compared to a fully IVEP embryo (Ferré et al. 2020).

In the wake of the genomic era, the field of in vitro embryo production is also impacted by transcriptomic and epigenetic tools. Genomic tools allow analyzing embryos at different stages, which provided a new avenue to compare in vivo and in vitro embryos.

11.4 World Scenario of Embryo Production Through OPU-IVEP

Globally it is observed that there has been a sharp rise in OPU-IVEP embryos during the last 5 years, with a remarkable jump in production during 2017, where a rise of 47.18% was seen from 2016. The International Embryo Technology Society (IETS) Data Retrieval Committee collects worldwide embryo production and transfer activity data annually. Data from the compilation from the last 20 years is presented in Table 11.3.

Worldwide production of OPU-IVEP embryos has shown dramatic shifts during 1999, 2003, and 2017 where production was almost doubled from the previous years. Currently, two countries are leading OPU-IVEP embryos production, i.e., USA and Brazil. The USA being the world leader in embryo production, experienced a 145% rise in embryos production in the last 5 years which is phenomenal (Viana 2020). During the year 2017, the USA surpassed the production of Brazil. Since 2002, Brazil has continuously shown an increase in OPU-IVEP embryos production, with the first decrease in 2019. The meteoric rise in in vitro embryo production in these countries is largely contributed by improvement in culture systems, large-scale availability of sex-sorted semen doses, and development of

Table 11.3 OPU-IVEP embryos production during last 20 years across globe (adopted from annual compilation of IETS)

Year	Total embryos produced	Embryos transferred		
		Fresh	Frozen	Total
2000	1,40,272	27,897	13,794	41,691
2001	1,09,205	15,379	14,885	30,264
2002	1,60,695	66,951	16,378	83,329
2003	3,41,730	91,372	14,848	1,06,220
2004	3,19,086	1,28,951	1,10,862	2,39,813
2005	3,30,647	1,83,477	82,514	2,65,991
2006	4,41,364	2,26,077	65,768	2,91,845
2007	4,34,581	2,15,512	29,745	2,45,257
2008	3,30,953	2,27,800	26,914	2,54,714
2009	3,76,576	2,83,188	22,761	3,05,949
2010	4,50,549	3,15,715	23,970	3,39,685
2011	4,53,471	3,43,927	29,942	3,73,869
2012	4,43,533	3,48,238	36,761	3,84,999
2013	5,17,587	3,58,440	35,185	3,93,625
2014	5,90,359	2,96,666	68,061	3,64,727
2015	6,12,709	3,04,946	99,227	4,04,173
2016	6,66,215	3,26,623	1,21,490	4,48,113
2017	9,80,524	4,92,848	2,56,766	7,49,614
2018	10,18,163	5,41,615	1,98,387	7,40,002
2019	10,10,680	4,38,476	3,47,663	7,86,139
2020	11,32,773	5,24,494	3,45,761	8,70,255

allied industries (such as equipment and consumables required for embryos production). It is also notable that the transfer of fresh OPU-IVEP embryos was preferred historically, possibly due to less cryo-tolerance. However, there is a rise in frozen OPU-IVEP embryo transfers, largely due to improvement in culture conditions and freezing methods, which contributed to the production of embryos equivalent to their in vivo counterparts.

11.5 Genomics and OPU-IVEP: A Whole New Perspective

With the advent of large-scale genomic selection programs, genomic breeding values or economic indexes are now available for newborn calves and biopsied embryos. This opened a new avenue for OPU-IVEP. Once the genetic worth is known at birth, OPU-IVEP can be planned as early as 4 months of age, if not even earlier than that. Similar demands from the customer are on the rise and keeping pace with the same industry is also equipping, like the development of smaller probes for heifers or laparoscopic OPU in calves (Landry et al. 2016). This allowed the production of calves from a high merit female at a very early age leading to a significant reduction in generation interval, thus contributing to an increased response to selection. Biopsy of embryos at different ages and estimation of genomic breeding values can further improve genetic gain by selecting the embryos expected to produce elite calves. The only issues with this are reduced pregnancy rate with biopsied embryos, chances of contracting diseases once zona is compromised, and restriction of international trade of biopsied embryos due to sanitary risk. However, with the development in vitrification, the pregnancy rate with biopsied and vitrified embryos has improved. Multiple programs were launched across the globe to transfer biopsied bovine embryos such as EliteOva, EmbryoGENE, etc.

11.6 Epilogue

The pregnancy rate is never similar with cryopreserved OPU-IVEP embryos compared to their in vivo counterparts. With the rise in the use of OPU-IVEP embryos, it is necessary to develop more efficient culture media, culture system, and freezing methods for the successful propagation of this immensely potential tool. Thus, large-scale funding is required for research and development to develop a detailed idea about different aspects of the technology. Integration of OPU-IVEP systematically in genetic improvement programs has not happened in many countries. Performing OPU-IVEP in isolation and producing embryos would not lead to the full realization of the potential of the technology. OPU-IVEP should be used to produce a large number of calves of desired sex to increase the selection intensity in the genetic improvement programs.

Further integration is also required in the production of calves from early age heifers to reduce generation interval. Evaluation of embryo quality in bovines is still largely dependent upon the visual grading of embryos using a microscope. At the

same time, the area has developed quite fast in the human IVF industry with induction pre-implantation testing of embryo biopsies, time-lapse incubators, or artificial intelligence in embryo grading. Large-scale research is in demand in the bovine industry to improve pregnancy outcomes using better-selected embryos.

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Milestones and Recent Developments in Farm Animal Cloning

12

Naresh L. Selokar, Manoj Kumar Singh, Dharmendra Kumar, Prem Singh Yadav, and Manmohan Singh Chauhan

Abstract

Somatic cell nuclear transfer (SCNT), popularly called "animal cloning (AC)," is the only assisted reproductive technique that allows reprogramming of a differentiated somatic cell genome to a totipotent embryonic stage which can result in a new life. Since the birth of the Dolly in 1996 (the world's first cloned farm animal produced from a differentiated adult somatic cell), many technical and scientific developments have been made in AC research worldwide, and over the years, several farm animal species such as cattle, goats, sheep, buffalo, pigs, horses, and camels have successfully been cloned. AC methods have opened many new avenues for agriculture and human biomedicine. The five major AC applications are (1) production of elite stock of farm animals, (2) conservation of endangered breeds or species, (3) production of artificial genetically modified (transgenic or edited) animals, (4) xenotransplantation to develop human organs, (5) nuclear-transfer stem cell lines for basic and clinical research. Despite potential applications, it has been debated and shown concerns about long-term use and food safety of products from cloned animals. In 2008, the world's regulatory agencies like the U.S. Food and Drug Administration (US-FDA) agreed that food (milk and meat) and food products derived from cloned animals and their offspring are safe and could not pose any extra risk to human health. Also, there is no need for any kind of labeling to clones and their offspring products for commercial use. However, low success rates, high cost, long-drawn-out regulatory processes, lack of awareness, and public disfavor have restricted the use of farm animal cloning worldwide. This book chapter aims to briefly discuss

N. L. Selokar (✉) · M. K. Singh · M. S. Chauhan
ICAR-National Dairy Research Institute, Karnal, Haryana, India

D. Kumar · P. S. Yadav
ICAR-Central Institute for Research on Buffaloes, Hisar, Haryana, India

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the milestones and recent developments in farm animal cloning as well as challenges and future prospective applications.

Keywords

Cloning · Buffalo · Elite animal production · Animal conservation

12.1 Animal Cloning: Fiction Stories to Farm Realities

Animal cloning (AC)—the method of creating identical copies of a living organism, particularly farm animals, has been a part of fiction stories in novels, movies, and mythological tales. The identical copies are called "clones." The AC, here specifically refers to somatic cell nuclear transfer (SCNT), allows the production of animals from somatic cells (not the reproductive cells, sperm, or oocytes) using an asexual way of reproduction. It means that the whole stretch of genetic material (DNA) is identical between parents (donors) and offspring (progenies). In principle, the AC is similar to a primitive type of printing technology that allows the production of a large number of duplicate reproductions of an original copy. Over the decades and with the development of science, the AC is now farm and lab realities. Several species of farm animals such as cows, buffaloes, sheep, goats, horses, pigs, camels, and pets like dogs, and cats have successfully been cloned worldwide (Keefer 2015).

Historically, the celebrated sheep, named "Dolly," was not the first farm animal produced through AC methods, few cattle and sheep were cloned using embryo splitting and blastomere cloning methods (Keefer 2015). Both embryo splitting and blastomere cloning would allow genetic improvement in farm animals. However, in embryo splitting, the production of a large number of embryos is a challenging and tiresome task, whereas in blastomere cloning, donor cells do not come directly from a live adult animal, but from early-stage embryos which demands stringent laboratory conditions. On the other hand, somatic cells are easy to culture and available in abundant numbers. To produce "Dolly," researchers used similar blastomere cloning method (micromanipulator-based) in which a differentiated somatic cell from an adult sheep was used as a nuclear donor instead of an embryonic blastomere cell (Wilmut et al. 1997). Before "Dolly," it had been opinioned that the reversal of cellular differentiation is biologically impossible; therefore, Dolly's birth had disapproved of the central dogma of developmental biology (totipotent embryo to germ layer cells to differentiated cell types such as muscle cells, blood cells, nerve cells, etc.). This landmark achievement has received great scientific attention and publicity. The studies that reported the first live cloned birth using differentiated somatic cells in farm animals are listed in Table 12.1.

Table 12.1 Milestones in animal cloning research

Publication year	Species	Name of the cloned animal(s)	Donor cell (s)	Country	Reference
1997	Sheep	Dolly	Adult mammary epithelial cell	United Kingdom	Wilmut et al. (1997)
1998	Cow	Twins, named Kaga and Noto	Adult cumulus and oviductal cells	Japan	Kato et al. (1998)
1999	Goat (cloned + transgenic)	Millie	Fetal fibroblast cell	USA	Baguisi et al. (1999)
2000	Pig	Five piglets, named Millie, Christa, carrel, dotcom and Alexis.	Adult skin fibroblast cells	USA	Polejaeva et al. (2000)
2002	Cat	CC (carbon-copy)	Adult cumulus cell	USA	Shin et al. (2002)
2003	Horse	Prometea	Adult skin fibroblast cell	Italy	Galli et al. (2003)
2005	Dog	Snuppy	Adult skin fibroblast cell	South Korea	Lee et al. (2005)
2007	Swamp Buffalo	–	Fetal fibroblasts and adult granulosa cells	China	Shi et al. (2007)
2009	Riverine Buffalo	Samrupa (Murrah buffalo)	Newborn skin fibroblast cell	India	Shah et al. (2009)
2017	Camel	Injaz (one-humped camel)	Adult cumulus cell	United Arab Emirates	Wani et al. (2017)
2018	Monkey	Twins, named Zhong Zhong and Hua Hua	Fetal fibroblast cells	China	Liu et al. (2018)

Listed milestones are the world's first report on the birth of the cloned animals

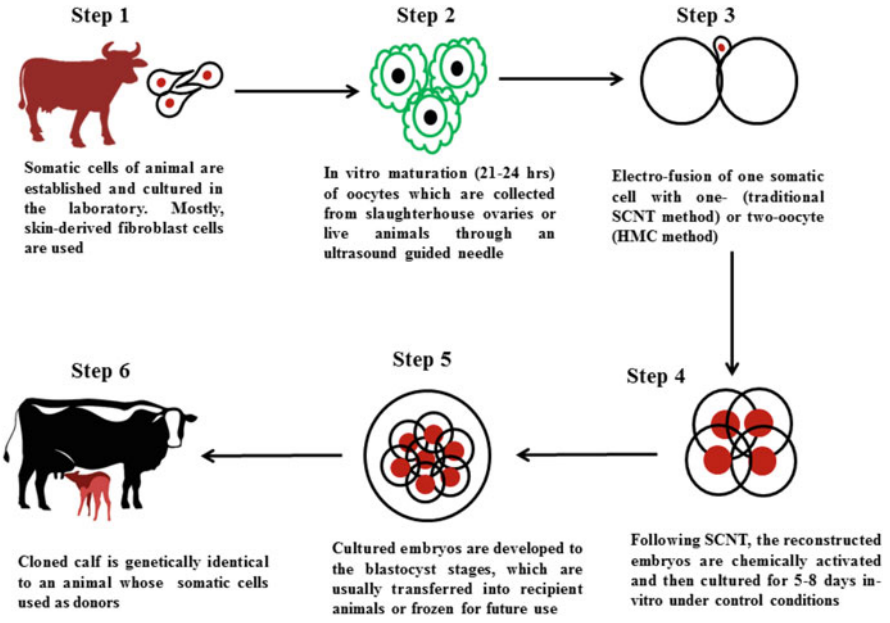

Fig. 12.1 An illustration showing the major steps of animal cloning [scientifically called somatic cell nuclear transfer (SCNT)] methods. HMC stands for handmade cloning

12.2 AC Methods

There are two basic biological requirements for AC—donor somatic cells and enucleated mature oocytes. The somatic cells are usually cultured from skin-tissue biopsies of animal(s) to be cloned, and cultured cells are propagated in the laboratory. Immature oocytes are collected from slaughterhouse ovaries and/or live animals and undergo in vitro maturation for 21–24 h. Following the maturation, oocytes are processed for enucleation (removal of the nucleus), and one or two enucleated oocytes are fused with one somatic cell using optimized electric current settings. The fused oocytes are then activated chemically to stimulate embryonic cell division. Following the activation, reconstructed embryos are cultured for 5–8 days (according to species) under in vitro controlled conditions in a CO_2 incubator to enable them to reach blastocyst-stage embryos. Blastocysts are then transferred non-surgically (cattle, buffalo, horses, and camels) or surgically (pigs, sheep, and goats) to recipient animals (surrogate mothers) to establish cloned pregnancies. After completion of the gestation period, clones are born which are genetically identical to donor animal(s) whose somatic cells are used in AC methods. We illustrate the important steps of AC methods in Fig. 12.1.

Two AC methods have been used widely to produce clones of animals. First one is a historic method called classical SCNT in which manipulation of oocytes are

Table 12.2 Classical SCNT vs simplified handmade cloning

Condition	Classical SCNT	Handmade cloning
Use of micromanipulator	Yes	No
Zona-free	No	Yes
Manual enucleation	No	Yes
Activation and culture methods	Similar	Similar
Problems associated with zona hatching	Yes	No
Problems associated with mitochondrial heteroplasmy	Less	High
Comparative cell number in produced blastocysts	Less	High
Problems associated with genomic reprogramming	Yes	Yes
Skilled manpower to perform experiments	Yes	No
Involved cost	High	Less

SCNT stands for somatic cell nuclear transfer. The table is acquired from Saini and Selokar (2018a)

performed using micromanipulator instruments (Wilmut et al. 1997), whereas the second method is called handmade cloning (HMC) in which manipulation of oocytes are performed under a stereo zoom microscope without the use of micromanipulators (Vajta 2007). Both methods have been used to clone the farm animals worldwide; however, the HMC can be preferred over classical SCNT due to the low cost of embryo production and less requirement of skilled workforce to perform oocyte/embryo manipulations. The major differences between classical SCNT and HMC are shown in Table 12.2.

12.3 AC Applications

AC is one of the assisted reproduction tools (ARTs) having potential applications in agriculture and human medicine (Fig. 12.2). The major applications of animal cloning are (1) production of elite stock of animals, (2) conservation of endangered breeds or species, (3) transgenic and genome-edited animals with improved production and/or health traits, (4) human organ development, mainly in pig, called xenotransplantation, (5) production of cloned embryos to establish embryonic stem cells (ESCs) for basic and clinical research. The emerging applications have led the establishment of commercial companies in the USA, China, Australia, South Korea, and Brazil for providing AC services to researchers, breeders, farmers, and pet owners.

12.3.1 Production of Elite Stock of Animals

About 10,000 years ago, the domestication of farm animals had been started. For domestication, equivalent wild animals were chosen based on food production traits (milk, meat), and other attributes such as captive breeding, disease resistance, and climate adaptability (Larson et al. 2014). About 200 years ago, the captive breeding

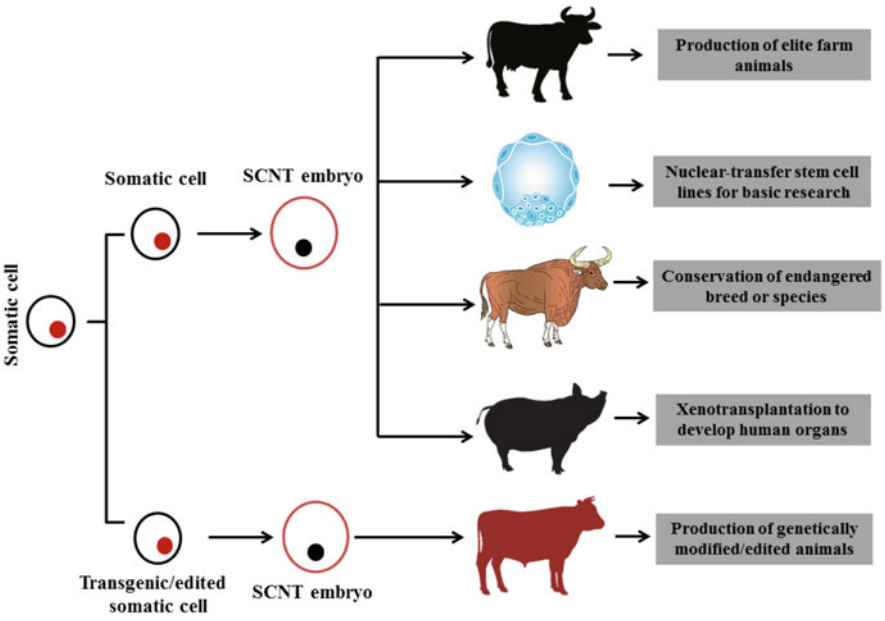

Fig. 12.2 Animal cloning applications. Animal cloning is mainly used to produce an elite stock of farm animals, and over the years, it also used to conserve the endangered breeds, produce transgenic/edited designer animals, develop human organs/cells in pig, and generation of patient-specific nuclear transfer stem cell lines. SCNT stands for somatic cell nuclear transfer

of acquired wild animals, mainly by natural mating, was practiced to develop well-defined breeds (Larson et al. 2014). About 100 years ago, the intensive selective breeding pressure, particularly with the use of artificial insemination, has further increased to develop the high prolific commercial breeds which we have today (Moore and Hasler 2017). With the help of selective breeding, genetics, and management, there is a dramatic increase in production traits (milk and meat), for example, milk production per cow has doubled in 40 years of selective breeding and high prolific cattle breeds produce more than 20,000 kg of milk per lactation (Oltenacu and Broom 2010). In the past years, improvements in production traits had been achieved through progeny testing programs, in which semen of high genetic merit bulls (based on dam's milk yield) are widely used for breeding, and also distributed worldwide to produce the high productive animals (Miglior et al. 2017). Recent advents in ARTs, omics (genomics, proteomics, transcriptomics, metabolomics, etc.), bioinformatics, and artificial intelligence technologies have launched a new era in farm animal breeding. Now, breeding goals are not limited to produce animals having high productivity for a single commodity (milk or meat), but also to improve disease resistance and climatic tolerance.

Classical selective breeding (CSB) could make desirable changes in the population; however, changes achieved at a very slow speed. CSB aided with ARTs such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET), in vitro

embryo production (IVP), and animal cloning (SCNT) could hasten the speed of genetic improvements (Moore and Hasler 2017). Among the ARTs, SCNT can bring genetic improvement at the fastest possible rate by producing a large number of high merit animals (having high milk and/or meat productivity, climate tolerance, and disease resistance traits) in the shortest possible time. The skillful application of SCNT could have an immediate effect on the animal production system and have a permanent effect on future animals which would help to meet the food demand of the continually growing human population, which is expected to be 9 billion by 2050 (FAO 2009). Therefore, there is a need to explore the application of AC in animal production systems to produce resilient, efficient, and productive farm animals. For example, the multiple copies of high merit animals (males or females) can be produced through SCNT and cloned elite copies to be used subsequently in other ARTs (AI, MOET, IVP) which would allow the large scale production of animals having unique genetic traits (Polejaeva et al. 2013).

Several studies have been reported that cloned animals have normal physiology and reproductive characteristics (Enright et al. 2002; Heyman et al. 2004; Shiga et al. 2005; Tecirlioglu et al. 2006; Smith et al. 2007; Ortegon et al. 2007; Polejaeva et al. 2013; Selokar et al. 2019a, b). In these studies, the reproductive performance of males (semen parameters and fertility) and females (ovarian cyclicity, oocyte recovery, embryo development, conception rates, and gestation period) were reported normal. Clones, that attained the maturity, have exhibited normal growth, health, blood hematology, serum biochemistry, and reproductive performance similar to non-cloned age-matched control animals or donors. Furthermore, the offspring of clones have exhibited normal phenotype, physiology, and reproduction, and production characteristics (Ortegon et al. 2007; Polejaeva et al. 2013). Therefore, the use of cloned animals for breeding cannot pose any extra risk to next-generation progeny.

In 2008, a risk assessment study published by the U.S. Food and Drug Administration (US-FDA) deemed that cloned and their offspring products (milk and meat) are safe for human consumption and practically indistinguishable from animals that are produced through conventional breeding (US-Food and Drug Administration (2008) Animal cloning: a risk assessment. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AnimalCloning. Accessed on 1 Jun 2020). Also, there is no evidence to suggest that consumption of food products from healthy clones and/or their offspring could pose an extra risk to human health. According to FDA guidelines, there is no need to tag the product of clones and their offspring for their source of origin. Also, there are no restrictions on the import or export of semen or embryos from cloned animals. Therefore, it is impossible to calculate the actual number of offspring produced by using semen/oocytes/ embryos of cloned animals. Despite potentials, the use of animal cloning is limited to produce breeding stock animals, but not the direct food source animals, this is mainly because of the low success rate and high cost. Keeping this in mind, clones of high merit animals can be placed in breeding programs aided with AI, IVP, MOET to increase the population of quality animals, wherein clone(s)' offspring become the actual food-producing animals. Figure 12.3 illustrates the application of AC in farm animal breeding. The readers

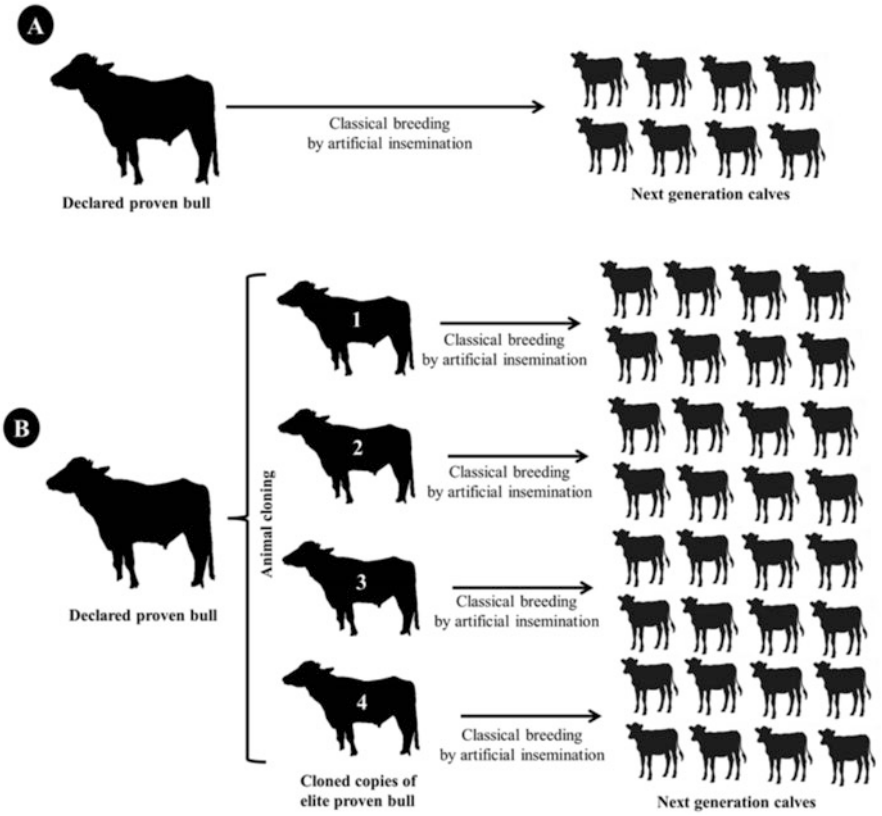

Fig. 12.3 Application of animal cloning in the animal breeding program. In the classical breeding program (A), the proven bull(s) are used to produce next-generation offspring using artificial insemination (AI). In the animal cloning-based breeding program (B), the cloned identical genetical copies of proven bull(s) to be used to produce a large number of offspring using assisted reproduction tools (AI, IVF, MOET). This way, genetic improvement can be achieved at a very fast rate as well as multiple locations or herds can be targeted. IVF stands for in vitro fertilization, MOET stands for multiple ovulation and embryo transfer. The figure is acquired from Selokar et al. (2018c)

can also follow a systematic review (Bousquet and Blondin 2004) in which the potential uses of AC in dairy breeding schemes have been discussed.

12.3.2 Conservation of Endangered Farm Animal Breeds

With the initiation of global trade, the commercial breeds have been exchanged among countries for their use in crossbreeding programs (mating of two or more breeds to create a new breed) to improve the production of low productive breeds from the same species (McAllister 2002). However, the problem is that commercial

breeds are well suited to the environment and management conditions of the original geographical location, therefore, high prolific breeds or crossbreds may not perform and survive well in the importing geographical location having different environments (FAO 2012). Despite crossbreeding has helped to improve production potentials of many breeds, it also poses an extra risk of extinction to locally adapted breeds. Over the decades, a rapid and continuous decline of farm animal breeds has been registered, whereby over 7000 livestock breeds are at risk of extinction worldwide (FAO 2012). Although, conservation strategies, both in situ *and* ex situ programs, are in place to rescue the population of endangered breeds to protect biodiversity, environment, farming, history, and economy; however, the endangered breed list is in the state of continuous upsurge (FAO 2012). In situ conservation strategies involve the active breeding of endangered animals within their habitat or production systems. However, in situ conservation is not enough when the population of a breed is reduced severely or when a breed habitat located in an unprotected environment (Holt and Pickard 1999). As a result, several ex situ conservation strategies have been developed involving in vivo conservation (maintenance and breeding of live animals away from natural habitat) and in vitro conservation (cryopreservation of sperm, oocytes, embryos, and tissues, somatic cells for potential future use through ARTs including animal cloning to revive the breeds) (Andrabi and Maxwell 2007).

Among biological materials stored for in vitro conservation, tissues and somatic cells ensure several advantages such as (a) tissue biopsies can be obtained easily from both male and female sex animals (sperm or oocytes preserve only one sex), (b) tissue biopsies can be obtained or transported from remote areas without specific requirements, (c) somatic cells can be cultured from old or dead animals, (d) tissues and somatic cells can be easily cryopreserved in circumstances where gametes and embryos are not technically feasible to cryopreserve or have very low success rates of cryopreservation (Selokar et al. 2018a, b). Also, the establishment of somatic cell/tissue biobanks is easy and cheap which makes them a very attractive option for cryoconservation. Somatic cell/tissue biobanks have significance for those countries, which have many breeds and/or limited resources like India (owns more than 150 breeds of farm animals—cattle, buffalo, goats, sheep, and pigs). Somatic cell biobanks are available in India (Buffalo, Selokar et al. 2018a, b), Vietnam (Cattle, Groeneveld et al. 2008), and Turkey (Cattle, Arat et al. 2011). And, it has been proved that bio-banked somatic cells can be used to produce cloned animals and/or embryos using AC methods (Arat et al. 2011; Selokar et al. 2018a, b).

AC methods provide an unprecedented opportunity to re-establish endangered breeds; however, limited availability of oocytes (starting biomaterial for SCNT) due to the low population of an endangered breed, it is a challenging task to effectively execute the use of SCNT to re-establish the breed. Therefore, inter-breed or inter-species SCNT (iSCNT) in which a somatic cell of an endangered breed/species is fused with an enucleated oocyte of another breed/species of which oocytes are available in large numbers for manipulation (Fig. 12.4). For example, somatic cells of India's Punganur breed of cattle can be fused with enucleated oocytes of other cattle breeds which are available in abundant numbers such as Sahiwal, Gir,

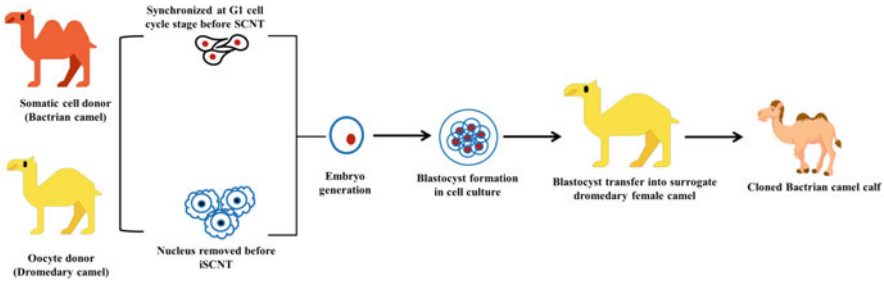

Fig. 12.4 Successful interspecies cloning in camels: The cloned Bactrian camel calf was produced by interspecies somatic cell nuclear transfer (iSCNT) in which somatic cells of a Bactrian camel was fused with an oocyte of a Dromedary camel which also served as a surrogate mother (world's first iSCNT cloned camel produced by Wani et al. 2017)

and Tharparkar. In previous studies (Lanza et al. 2000; Loi et al. 2001; Gomez et al. 2004; Wani et al. 2017), the encouraging iSCNT results have been obtained in some endangered mammal species such as Gaur (*Bos gaurus*), European mouflon (*Ovis orientalis*), African wild cat (*Felis silvestris lybica*), and recently Bactrian camel (*Camelus bactrianus*). These outcomes have opened a ray of hope to re-establish endangered breeds/species through the application of iSCNT. Therefore, there is an urgent need to collect and cryopreserve tissue biopsies and/or somatic cells from endangered breeds for short- and long-term storage in biobanks. In case, the population of any particular breed collapse or vanish, the bio-banked somatic cells or tissues can be used promptly to re-establish the breed in the population using iSCNT.

12.3.3 Production of Genetically Modified (Transgenic or Edited) Animals

Traditionally, the genetic changes in the genome of farm animals have been achieved through selective breeding, in which continuous mating of favorable elite animals with unfavorable animals over many generations leading to up-gradation of specific alleles (Hale 1969). Over the last 30 years, genetic engineering (GE) has been evolved in such a way that the genetic information (DNA) can be manipulated (remove, add, or modify) into any living organism to improve existing or introduce novel genetics within one generation. The employment of recently developed GE tools [Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALEN), and clustered regularly-interspaced short palindromic repeats (CRISPR)] would save the eight to ten generations of back-crossing required in conventional breeding to introduce a specific allele. In Fig. 12.5, we illustrate the comparison between traditional breeding methods and current GE methods, both can be used to produce desired animals. Before the birth of famed "Dolly," the world's first genetically modified bovine, Herman the Bull, was produced by manipulation of an early-stage embryo with the human lactoferrin gene (Krimpenfort et al. 1991).

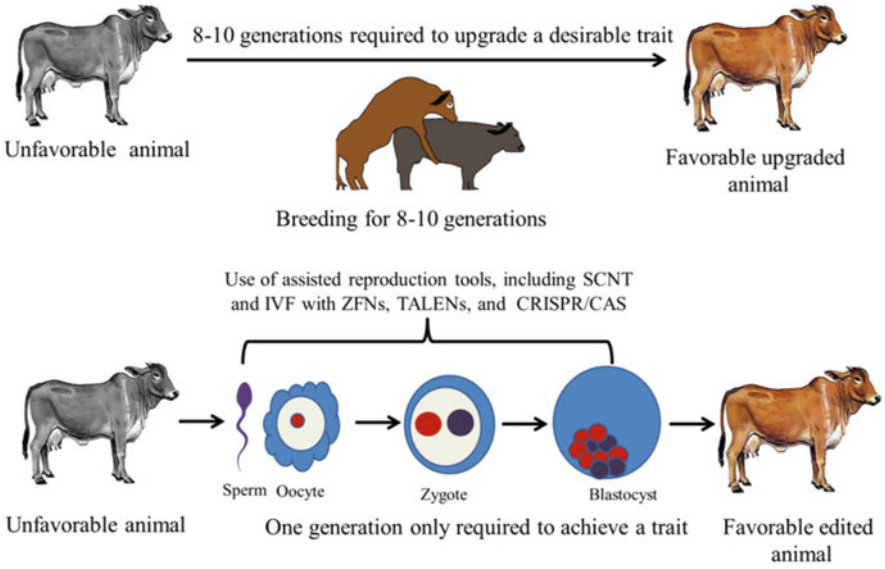

Fig. 12.5 Comparison between traditional breeding methods and current evolved genetic engineering methods. Using the application of genetic engineering methods, a desirable trait can be achieved in one generation

This bull was sired of 83 calves that inherited the lactoferrin gene (Seltzer 1994). This study opened the unlimited promises aiming to manipulate gene(s) to create designer animals for agriculture and human medicine such as (1) manipulation of genes to improve the milk and meat production, (2) production of therapeutic proteins in milk, urine, or blood, (3) animals with improved disease resistance, (4) environmental friendly animals, and (5) model animals for biomedical or veterinary applications. Multiple successes have already been demonstrated by using GE tools aided with AC methods (reviewed by Tan et al. 2016). However, wide-scale applications have always been a challenge due to the extremely poor efficiency of both GE tools and AC methods.

In the recent 5 years, GE has acquired a great momentum due to the discovery of engineered nuclease, namely CRISPR (popularly called genome scissors). Among the engineered nucleases, the CRISPR system has been extensively used to manipulate the genomes due to its simplicity, specificity, and efficiency. In principle, engineered nucleases create double-strand breaks (DSBs) at specific sites in the genomic DNA. DSBs are repaired either by the non-homologous end joining (NHEJ) or the homologous directed repair (HDR) procedures, which have been exploited to manipulate the genomes (reviewed by Gaj et al. 2013). NHEJ is an error-prone procedure involving the interrupted joining of the broken ends of DNA that creates insertions and deletions (indels) of nucleotides at cleavage sites leading to disruption of gene function. HDR is a more accurate procedure that requires homologous DNA sequences as templates for repair; therefore, the desired

Table 12.3 Selective examples of genetically modified farm animals produced using engineered nucleases

Phenotype	Target gene	Species
Hornless cattle	Polled locus	Cattle
Increased resistance against bovine tuberculosis	NRAMP1	Cattle
Resistance against porcine reproductive and respiratory syndrome virus infection	CD163	Pig
Increased muscle mass	Myostatin	Ruminants
Germline ablation	NANOS2	Pig

The table is acquired and modified from Petersen (2017)

nucleotide sequence can be integrated into the genome by supplying it as an exogenous DNA template. Few noticeable examples of genetically modified farm animals produced using engineered nucleases are listed in Table 12.3.

Efforts are ongoing to improve the nutritional value of milk, either by knock-out of the beta-lactoglobulin (dominant milk allergen) or by expressing lactoferrin (a glycoprotein supports the absorption of iron from the intestine and suppresses the growth of bacteria). Also, attempts had made to express human lysozyme into the mammary gland of cows (Liu et al. 2014) to protects cows from mastitis (the mammary gland disease responsible for economic loss in dairying). In pigs, a RELA gene had manipulated to produce genetic resistance to African swine fever, an endemic disease of the swine industry (Lillico et al. 2013). For more muscle growth, myostatin is a common target gene to produce double-muscled mass farm animals (Fiems 2012). For other exciting examples, the readers can follow recent exhaustive reviews on genetic engineering in farm animals (Petersen 2017; Tait-Burkard et al. 2018; Kumar and Kues 2020).

The landmark success in the field of genetically modified (GM) animals has achieved in the year 2017. The US-FDA and the Canadian Food Inspection Agency have approved genetically modified Salmon fish (an only transgenic animal) for human consumption (Tait-Burkard et al. 2018). To achieve the long-term impact, the well-established and proven GE protocols, which successfully demonstrated and produced model designer animals, need to be translated efficiently into farm animal breeding and human medicine. It is also likely that soon several edited animals will be produced; however, editing/transgenic concepts and proofs have to be ethically accepted and approved.

12.3.4 Xenotransplantation Attempts to Develop Human Organs in Pigs

Shortage of organs/cells for transplantation in human patients having end-stage organ failure has led to the development of artificial organs/cells and

Table 12.4 Comparison of farm animal (pig) with nonhuman primate (Baboon) for xenotransplantation research

Feature		Baboon	Pig
Clinical aspect	Anatomical similarity to humans	Closed	Moderately close
	Physiological similarity to humans	Closed	Moderately close
	Relationship of immune system to humans	Closed	Distant
	Risk of transfer of infection (xenozoonosis)	High	Low
	Knowledge of tissue typing	Limited	Moderate
Experimental aspect	Availability	Limited	Unlimited
	Breeding potential	Poor	Good
	Period to reproductive maturity	3–5 years	4–8 months
	Number of offspring per pregnancy	1–2	5–12
	Growth	Slow	Rapid
	Size of adult organs	Inadequate	Adequate
	Cost of maintenance	Very high	Low
	Experience with genetic engineering	None	Considerable
	Public opinion	Mixed	More in favor

The table acquired and modified from Gianello (2014)

xenotransplantation (animals to humans transplantation). Xenotransplantation involves the transfer of body fluid, cells, tissues, or organs from live-nonhuman animals, mainly nonhuman primates and pigs, to humans to improve or save the patients' lives (Xenotransplantation, WHO, Geneva, Switzerland. Available at URL: http://www.who.int/transplantation/xeno/en/).

About 75 years ago, nonhuman primates had been considered as animal models for human organ donors. However, because of the potential risk of disease transmission, long time, more primate breeding expenses, less availability of organs, and ethical issues; other potential donor animals had been sought (Cooper et al. 2016). Among the farm animals, the pig is a preferred animal choice to develop human organs/cells. Some of the logical and ethical advantages to pigs for xenotransplantation research are (1) readily available in various body sizes; (2) anatomical and physiological similarities to human organs; (3) fewer chances of diseases transmission; (4) large offspring produced per pregnancy; (5) easy at captive breeding; (6) ethically accepted. The differences between pigs and nonhuman primates are listed in Table 12.4. In the current experiments, pigs are used to develop human organs, and baboons are used as human models (Gianello 2014).

Despite several advantages; the operational transplantation of pigs' organs to humans has not been a reality due to two major issues such as graft rejection and potential cross-species infection (Gianello 2014). It has been found that hyperacute graft rejection was mainly due to the expression of a carbohydrate antigen, named galactose-α1, 3-galactose (αGal), in pigs. Humans lack the αGal epitopes but are

exposed to bacterial αGal epitopes that elicited the anti-αGal antibody response (Gianello 2014). As a result, when pig's organs/cells transplanted into humans, the anti-αGal antibodies bind immediately to transplanted organs/cells, and activate the complement cascade, resulting in graft rejection (Gianello 2014). Since it has been known that the αGal epitope causes the immune rejection, the researchers have focused efforts to develop pig models that lack the αGal antigen. It becomes possible to delete the αGal antigen gene using homologous recombination technologies and recently evolved GE tools aided with AC methods (Cooper et al. 2018). This is the foremost benefit of AC technology in pigs for overcoming barriers of xenotransplantation.

The AC allows genetic changes at the donor cell stage that results in desired founder pigs in the first-generation, whereas in other methods such as pronuclear microinjection or cytoplasmic injection, and blastocyst cell transfer can produce chimeric founder animals requiring subsequent selective breeding to generate homozygous pig lines (Cooper et al. 2018). One of the futuristic ideas is that iSCNT using human (having end-stage organ failure) cells and pigs' recipient oocytes to be used to generate the patient-specific nuclear transfer stem cell lines, and later these stem cells to be transplanted or fused with early-stage pig embryos, in which transplanted cells become an integral part of late-stage embryos (post-morula stage and blastocysts). Such tailored embryos would produce tailored pigs having human cells/organs, hoping that such designer organs would be suitable for transplantation in an intended patient. This will not be possible unless all hurdles of xenotransplantation are overcome. The increasing advances in GE tools and pig genomics will certainly make xenotransplantation future vibrant.

12.4 Therapeutic Cloning

Embryonic stem cells (ESCs) can be derived from blastocyst-stage embryos produced through IVF, parthenogenetic activation, or SCNT. ESCs properties such as unlimited self-renewal capacity and ability to differentiate into all body's cell types make them a very attractive option to replace specific cells in damaged organs/tissues and to stem cell-based research. To solve the problem of graft rejection of xenotransplantation, AC methods have been envisaged to produce patient-specific nuclear-transfer ESCs (NT-ESCs) for autologous transplantation called therapeutic cloning (Eguizabal et al. 2019). Besides the SCNT, the patient-specific SCs can also be produced through the forceful expression of transcription factors (such as Oct3/4, Sox2, Klf4, and c-Myc known as OSKM or Yamanaka factors) through viral vectors or plasmids in patient's somatic cells. This method of generating stem cells are called induced pluripotency; however, iPSCs have several limitations such as viral gene integration, incomplete erasure of epigenetic marks, and genetic instability (Eguizabal et al. 2019). These two pluripotent SCs generation methods, SCNT and iPSC, are illustrated in Fig. 12.6. SCNT methods have been used successfully to generate bona fide ESCs in mice, nonhuman primates, and humans. In 2013, a remarkable study demonstrated that human ESCs produced using the SCNT method

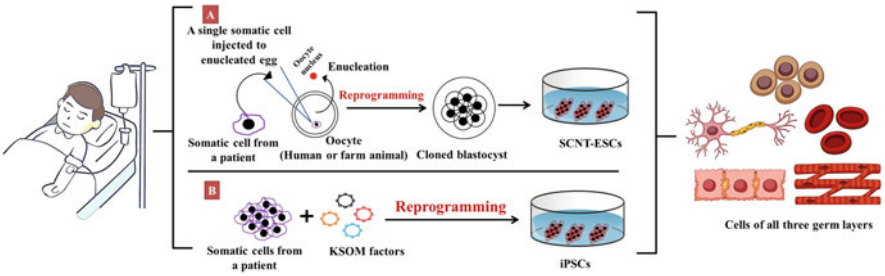

Fig. 12.6 The commonly used methods to generate patient-specific pluripotent stem cells. The first method is SCNT-based, in which the patient's somatic cells are injected into enucleated oocytes to produce blastocyst embryos to generate pluripotent embryonic stem cells (**a**). (**b**) The second method is transcription factors-based, in which the patient's somatic cells are transduced with transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) to generate pluripotent stem cells called induced pluripotent stem cells (iPSCs)

had displayed normal diploid karyotypes and the nuclear genome inherited exclusively from donor somatic cells. Also, gene expression and differentiation profiles in human NT-ESCs had similar to IVF embryo-derived ESCs (Tachibana et al. 2013). This study showed that the SCNT allows efficient reprogramming of human somatic cells to ESCs.

Due to the limited availability of human oocytes and the use of human oocytes could pose serious ethical issues, the farm animal oocytes (which are available in large numbers) can also be used to generate patient-specific NT-ESCs through iSCNT approach. To achieve these goals, Chung et al. (2009) conducted a study using cow and rabbit oocytes to reprogramme somatic cells of humans aiming to generate NT-ESCs. They produced human–human, human–bovine, and human–rabbit cloned embryos, and embryo gene expression profile showed that key pluripotency genes (Oct-4, Sox-2, and Nanog) had up-regulated in human-human cloned embryos as compared to human–bovine, and human–rabbit cloned embryos, suggesting inefficient reprogramming of human somatic cells by animal oocytes. It has been perceived that the feasibility of this approach to produce patient-specific NT-ESCs is yet to be demonstrated, and many cell reprogramming mechanisms remain unanswered, and more attempts are required.

12.5 Animal Cloning in India-Buffalo is the Only Farm Animal Species Successfully Cloned So Far

India has the huge farm animal population, 535.78 million heads in which 192 million cattle, 109 million buffaloes, 74 million sheep, 148 million goats, 9.06 million pigs (20th Livestock Census data published in 2019, accessed on June 4, 2020, at http://www.dahd.nic.in/about-us/divisions/statistics). Despite having several species of farm animals, buffalo has been preferred over others for AC applications. Some of

the reasons are (1) ample availability of oocytes for manipulations (cow oocytes are not available due to ban on cow slaughter), (2) most assisted reproductive techniques (embryo culture, IVF, MOET) are well optimized, (3) scientific expertise and technical personnel are available, including staff for efficient manipulation of oocytes and embryo transfer.

India possesses the best breeds of buffalo, particularly Murrah, which is famous all over the world for its ability to produce a high quantity of milk. India is currently the largest milk producer in the world. In 2018–2019, 187.75 million tonnes (mt) of milk were produced in India, of which buffalo's contribution was 91.82 mt (49%), which is more than the milk obtained from crossbred/exotic cattle (51.26 mt) and indigenous/nondescript cattle (38.57 mt) (Annual Report 2018–2019, Department of Animal Husbandry & Dairying, GOI, New Delhi; accessed on June 5, 2020, at http://www.dahd.nic.in/documents/reports). Therefore, India's white revolution cannot be imagined without the contribution of buffalo. To sustain this level of milk production, elite high milk-producing animals need to be multiplied at a fast pace through scientific interventions.

The first animal cloning attempt in buffalo was made by researchers at the ICAR-National Dairy Research Institute (NDRI), Karnal during the late 1990s (Singla et al. 1997). Cells from in vitro fertilized or in vivo produced embryos (called blastomeres) were injected into enucleated oocytes to generate cloned embryos. The method used during that time was similar to Dolly's method having use of micromanipulator instruments but could not produce blastocyst-stage embryos. Later, in the early 2000s, NDRI researchers have acquired the economical, safe, and efficient HMC method. In 2009, the NDRI has created history in the field of AC by producing the world's first cloned riverine buffalo calf, named "Samrupa." Over 25 buffaloes have been cloned using different donor cell types such as embryonic stem cells, urine-derived epithelial cells, semen-derived epithelial cells, etc. In 2015, the NDRI's buffalo cloning recipe had replicated successfully by researchers of the ICAR-Central Institute for Research on Buffaloes (CIRB), Hisar. And, CIRB becomes India's second institute that produced a cloned buffalo. For a detailed journey of buffalo cloning in India, the readers can follow reviews published by us (Selokar et al., 2018a, b, 2019a, b; Saini et al. 2018b). Available live buffalo clones in India are listed in Table 12.5. To date, only two Indian institutions, NDRI and CIRB, have all infrastructure and skilled manpower for producing cloned buffaloes, and the current aim is to produce multiple copies of elite breeding bulls, mainly Murrah.

12.6 Challenges in AC Applications

There is no doubt that AC is a powerful tool providing many opportunities for farm animal production, conservation of endangered breeds/species, and human medicine. However, AC is suffered from several challenges such as (1) poor success rates (only 5–10% of embryos transferred into recipient animals could result to healthy surviving clones, whereas in other ARTs (AI and IVF), 30–40% results following

Table 12.5 Available buffalo clones in India as on May 2022

Sex of donor	Age of donor	Cell type	Month of birth	Birth weight (kg)	Current status
Female	Embryonic stem cell	IVF embryonic stem cell	August 2010	32	Delivered calves
	Adult	Skin fibroblast	May 2014	36	
	Adult	Urine-derived epithelial cell	February 2015	37	
Male	Fetal	Skin fibroblast	August 2010	41	Produced semen, and calves following artificial insemination in females
	Adult	Fresh semen-derived epithelial cell	March 2013	55	
	Adult	Frozen thawed semen-derived epithelial cell	July 2014	32	
	Adult	Tail-skin-derived fibroblast	December 2015	42	
	Adult[a]	Tail-skin-derived fibroblast	December 2017	54	Under semen production
	Six adult clones from a single donor animal	Tail-skin-derived fibroblast	October to December 2020	40–48	Under semen production
	Three cloned copies from a single donor animal	Tail-skin-derived fibroblast	November 2020 to February 2021	40–45	One clone is under semen production

Listed cloned buffaloes are produced using the handmade cloning method

[a]A breeding bull of Assamese buffalo was used as a donor and the cloned calf was born in the field (World's first report in buffalo); others are the clones of the famous Murrah buffalo. The table acquired and modified from Selokar et al. (2018a) and recent updated data. These clones are available at NDRI, Karnal and CIRB, Hisar

embryos transferred/semen insemination), (2) abnormal placental development, (3) loss of clones throughout gestation, at time of calving, and post-natal period (generally within 6–8 months of age), (4) prolonged gestation period in cloned pregnancies, and the birth weight of clones maybe 20–30% more than normal, (5) born clones require special care and management since they may succumb to infection and stress (Keefer 2015). Extensive research on genomic and epigenomic showed that these abnormalities are probably due to incorrect/improper genetic and

epigenetic reprogramming of a differentiated donor cell by a recipient oocyte that leads to abnormal gene expression throughout the development of clones (embryo to calf). Not all clones are abnormal, studies showed that once clones mature, they grow and reproduce similar to non-cloned animals (Polejaeva et al. 2013; Selokar et al. 2019a, b).

If AC to be a reliable technique and needs to sustain lifelong, it is expected that technology deficits should be minimized and the success rate should be improved so that it can work parallel with other ART tools. To achieve these goals, many modifications have been performed such as (1) selection of best donor cell type and stage of recipient oocyte, (2) treatment of donor cells/oocytes/fused embryos with epigenetic modulators, siRNAs and miRNAs, (3) modification in embryo culture conditions (at present, all types of ART embryos are cultured under lower oxygen tension), (4) formulation of best culture media (for example, buffalo cloned embryos develop well in K-RVCL media), (5) non-invasive approaches (metabolic makers in spent media) to select viable embryos before transfer to recipients, (6) genetic manipulation of donor cells using newly discovered editing tools (CRISPR) to correct epigenetic errors. However, the exact cause of faulty reprogramming is not yet identified, and in vitro improvements did not fully translate into in vivo success. More efforts are, therefore, needed to improve the live birth rate with cloned embryos. With advances in genomics and embryo culture techniques, it may expect that all reprogramming issues will be solved, and clones or their progeny would become farm realities.

12.7 Prospective and Perspective

With significant milestones and the build-up of scientific data, it has been perceived that AC is an invaluable research tool to produce elite animals, conservation of endangered breeds, xenotransplantation, and many more research opportunities. The deleterious climate change, emergence and re-emergence of disease pathogens, and non-availability of fodders are catching the attention of many scientists, how they can overcome some of these concerns through the intervention of AC for sustainable animal production and health. The AC methods aided with new gene-editing technologies (CRISPR), omics, and artificial intelligence could manage some of these concerns. Few examples which can be a future reality are as follows: (1) production of animals carrying disease resistance (e.g., foot-and-mouth disease (FMD), Rinderpest, and swine fever, etc.) or stress-tolerance (e.g., extreme heat or extreme cold) genes, such tailored animals would be used to produce healthy and tolerant offspring, (2) sexed semen producing bulls (only X sperm in semen) particularly for dairy farming in which the majority of males (except breeding bulls) become unprofitable to farmers due to mechanization of agriculture, (3) production of veterinary vaccines (e.g., FMD and Rinderpest) or human therapeutic proteins (e.g., insulin) into a mammary gland for clinical uses (ATryn, a commercial anticoagulant (anti-thrombin drug), is a first therapeutic protein secreted in the mammary gland of goats), (4) pig organs for human transplantation, (5) revival of old valuable and historical breeds to have the highest diversity ever.

Since the birth of "Dolly," ethical concerns have been discussed worldwide about the long-term use and food safety of animals produced through AC methods. In 2008, the US-FDA had published comprehensive guidelines for use of animal clones and clone progeny for human food and animal feed. According to FDA guidelines, the composition of milk and meat of clones and their progeny are similar to other animals produced through AI, IVP, and MOET. Therefore, food/feed products derived from clones or their progeny could not pose any consumption risks to consumers (humans and animals). It also anticipated that AC technology is mainly used to produce elite stock, and they will not directly use as food animals, but clone progeny (sexually reproduced offspring of clones) will act as actual food animals. Therefore, the FDA believes that food/feed products from clone progeny are suitable to enter the food and feed supply chain under the same regulatory controls which are applied to animals that produced using sexual reproduction. To date, expect European Union member states, most of the countries do not have any regulatory blockage on the use of AC technologies. Animal cloners wish and believe that AC technologies will complement other ARTs for producing profitable animals and reviving old historic breeds, and also help to solve the shortage of human organs and other prospective clinical research avenues for humans.

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Advances and Applications of Transgenesis in Farm Animals

13

T. R. Talluri and Bhanu P. Telugu

Abstract

Modification of animal genomes is an age-old tradition rooted in selective breeding of genetic outlier animals over successive generations for enhancing production traits. However, selective breeding has limitations; chief among them is the requirement for the existence of a beneficial trait within the population, low heritability of traits, and the long time required for mating over multiple generations to "fix" the trait within a population. Given the need to sustainably increase animal production to feed the anticipated 9 billion global population by 2030, and a looming threat from climate change, there is a pressing need for animal agriculture to be more precise and responsive than selective breeding. This includes utilizing all the tools at our disposal such as transgenesis and genome editing. The emergence of genome engineering tools like meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas (CRISPR/Cas) system allows us to introduce precise genetic modification at nucleotide resolution while also facilitating large transgene integration. Concordant with the developments in genomic sequencing, progress among transgenic approaches has reached feverish pace. The current manuscript reviews past and current developments in gene transfer techniques, including microinjection method, sperm mediated gene transfer, cytoplasmic DNA injection, cloning/somatic cell nuclear transfer (SCNT), embryonic stem cell, and retroviral vector

T. R. Talluri
Equine Production Campus, ICAR-NRC on Equines, Bikaner, Rajasthan, India

B. P. Telugu (✉)
Animal Sciences Research Centre, College of Agriculture, Food and Natural Resources, University of Missouri, Columbia, MO, USA
e-mail: telugub@missouri.edu

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mediated methods to improve efficiency of genetic engineering, while also focusing on novel genome editing tools like designer nucleases for the generation of transgenic animals. Cumulatively, these tools provide a better platform to produce transgenic animals for addressing critical priorities of animal agriculture and biomedical research.

Keywords

Transgenesis · Genetic modification · Methods · Applications · Farm animals

13.1 Introduction

Transgenic- or genetically engineered animals are the result of an inheritable genetic modification introduced by the artificial transfer of an exogenous DNA fragment or an intentional alteration of an endogenous DNA. The term "transgenic" was coined by Gordon and Ruddle (Gordon and Ruddle 1981) in the year 1981 to describe an animal in which an exogenous gene had been stably and deliberately incorporated into the genome. A genetically engineered or "transgenic" animal carries a known sequence of recombinant DNA in recipient animals and passes on the genetic modification to its progeny as a host gene. The term "transgenic" was also extended to gene knockout animals, where a gene of interest or a candidate gene has been deliberately knocked out or deleted from the genome (Beardmore 1997). This technology has immense promise for enhancing the quantity and quality of livestock products at a rapid pace compared to traditional and customized breeding methods in livestock. Earlier, experiments in animal transgenics were performed using mice as model, but as of today, many larger animals have been engineered, including livestock such as cattle, sheep, goats, and pigs, pets such as cats and dogs, and even non-human primates (Fig. 13.1) (Zhao et al. 2019). By and large, the farm animals were genetically modified for agricultural and biomedical applications, including but not limited to enhancing economically important production traits, and for use as bioreactors, disease models, xenotransplantation, and as preclinical models for translating findings to potential therapeutics (Fig. 13.2; Table 13.1). With the rapid improvement of gene sequencing platforms that are becoming progressively cheaper, it is now possible to identify the genotypes and alter the genomes of different livestock for desired function.

In a landmark experiment, the first transgenic animals were produced 35 years ago by microinjecting the exogenous DNA (Han and Park 2018; Kalds et al. 2019). Microinjection was the only preferred choice for generating transgenic animals for more than 30 years. However, alternative protocols have since emerged, including cloning/somatic cell nuclear transfer (SCNT), embryonic stem cell (ESC) modification and chimera generation, viral vectors, cytoplasmic DNA injection (CPI), and sperm mediated and testes mediated gene transfer (SM/TMGT) methods to name a few, for generating transgenic animals. Recent discovery and validation of genome editors such as meganucleases, ZFNs, TALENs, CRISPR/Cas systems provided

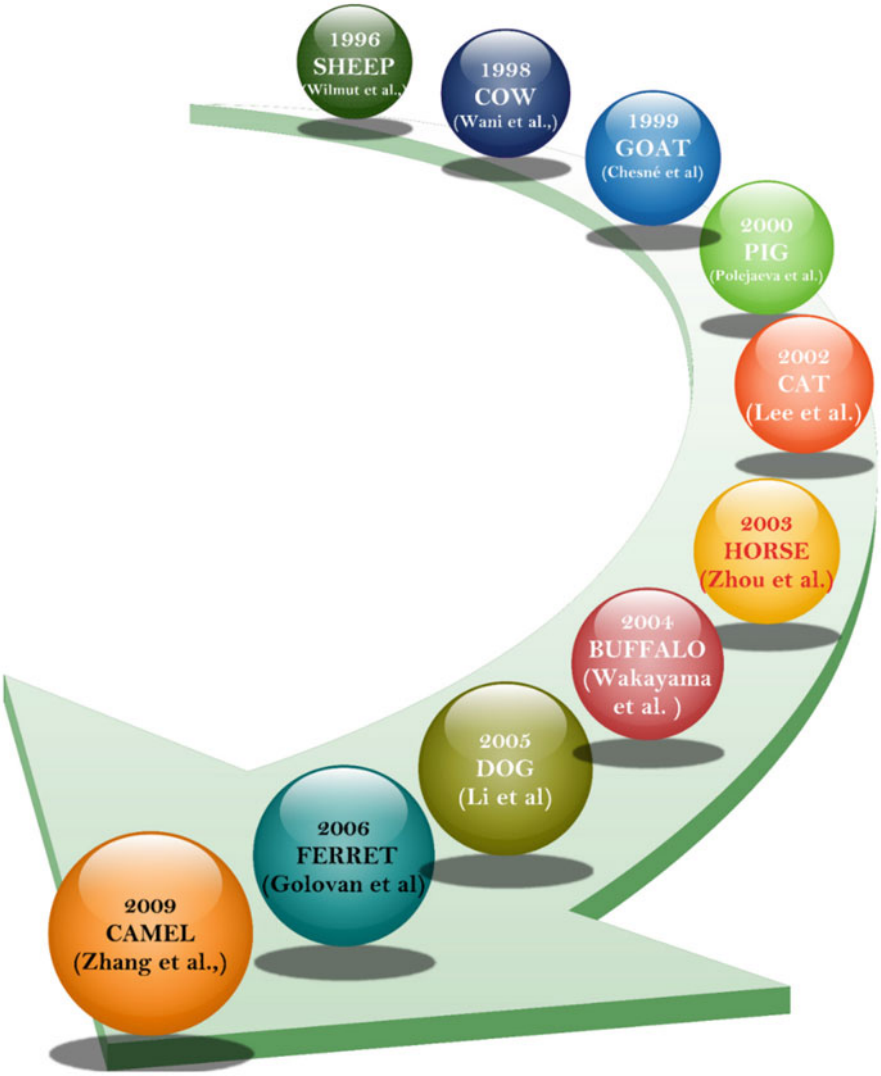

Fig. 13.1 Timeline of production of transgenic animals

further fillip to genetic engineering efforts. The editors which are designer nucleases induce double strand breaks (DSB) at a targeted genomic site, thereby triggering endogenous DNA alterations via either non-homologous end joining (NHEJ) or homology directed repair (HDR) pathways (Fig. 13.3). The repair mechanisms triggered by the DSBs can be leveraged for introducing targeted genetic modifications through genome editing (GE). The advent of GE dramatically altered the landscape of genetic modification in farm animals (Bosch et al. 2015; Petersen and Niemann 2015). GE is fundamentally different from conventional transgenesis

Fig. 13.2 Applications of transgenic livestock

Table 13.1 List of farm animals produced through cloning

Species	Donor cell type	Year	Reference
Sheep	Differentiated embryonic cell line	1996	Campbell et al. (1996)
	Adult mammary epithelium	1996	Wilmut et al. (1997)
Cow	Transgenic fetal fibroblasts	1998	Cibelli et al. (1998)
	Adult cumulus and oviductal cells	1998	Kato et al. (1998)
Goat	Fetal fibroblasts	1999	Baguisi et al. (1999)
Pig	Fetal fibroblasts	2000	Onishi et al. (2000)
	Granulosa cells from adult pig	2000	Polejaeva et al. (2000)
Buffalo	Fetal fibroblasts and adult granulosa cells	2007	Shi et al. (2007)

in that it allows for a precise and desired modification at the intended location within the genome. This technology has overcome some deficiencies associated with classical transgenesis such as random integration, positional variegation, and lack

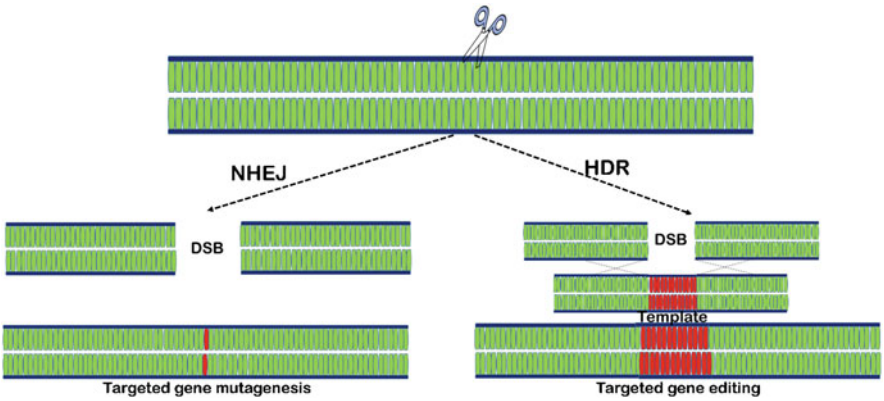

Fig. 13.3 NHEJ and HDR pathways

of expression (gene silencing), among others. In addition, GE is relatively facile, and faster, than transgenesis. These platforms in which genes can be selectively and systematically switched "on" or "off," or novel genes introduced further improve our options for GE in livestock species. In this article, we briefly review the customized methods used for producing the transgenic animals, briefly introduce the recent advances in nuclease-meditated gene editing tools, outline various applications of GE in livestock genomes, and briefly provide perspectives regarding the challenges and prospects of the new GE technologies.

13.2 Methods for Genetic Modification

Approaches for genetic modification can be classified into two broad categories:

1. *Embryo-mediated approaches*: In this approach, the target gene is modified in the embryo, and the resulting embryo is implanted into surrogates to generate the genetically modified founder animal.
2. *Cell-mediated approaches*: In this technique, the desired alteration is introduced into the cells, and the modified cell is used for producing the genetically modified animal via SCNT.

Both approaches are successfully employed for making transgenic animals and are discussed briefly below.

13.2.1 Embryo- Mediated Approaches

13.2.1.1 DNA Microinjection into Zygotes

In 1980, Gordon et al. for the first time produced the transgenic mice by microinjecting purified DNA fragments into the pronuclei of zygotes (Gordon et al. 1980). This technique involves assembly of a transgenic construct containing the genes of interest under the control of an appropriate promoter, recovery of the zygotes from donors, microinjecting the transgene into the male pronucleus, and implantation of microinjected zygotes into pseudo-pregnant recipient animals for the birth of transgenic founder animals (Fig. 13.4). The injected transgene randomly integrates into the embryonic genome, and the resultant founder animals invariably have varied number of integration sites, transgene copy numbers, and a frequent incidence of mosaicism (i.e., cells carry varying number of transgene copies), which together reduce heritability of transgenes in subsequent generations (Table 13.2). Therefore, a major drawback of this procedure is the requirement for the generation

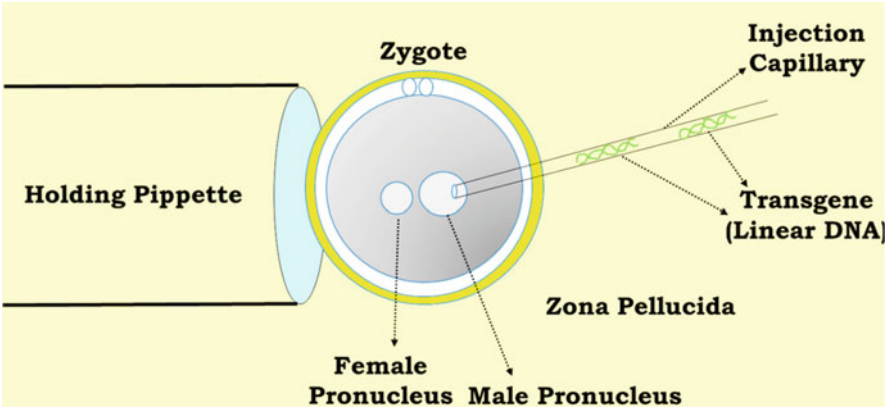

Fig. 13.4 Pronuclear microinjection technique

Table 13.2 Comparison between Microinjection and Somatic cell Nuclear Transfer (SCNT) techniques

Characteristic	Microinjection	Somatic cell nuclear transfer
Site of integration	Random	Can be targeted or random
Efficiency of integration	Weak	Very strong
Gene deletion	Not possible	Possible
Mosaicism	Very frequent	Rare event or less common
Multitransgenesis	Less common and feasible	Common and more feasible
Expression screening in vitro	Not possible or less common	Very much possible
Technical feasibility	Yes	Yes
Pattern of expression	Uncontrollable and variable	Consistent and controllable expression

of a greater number of founder animals to screen for transgene copy number integration, expression, and transmission to the next generation. Regardless, this method of generating transgenic animals remained the method of choice for more than two decades and has been proven to be tractable in species like goat (Freitas et al. 2007), rabbits, and sheep (Hammer et al. 1985). However, zygotic microinjection has proven to be impractical in the case of cattle (Eyestone 1999) and pigs due to difficulties in the visualization of male pronucleus (Perry et al. 1999; Salamone et al. 2018) due to high lipid content in the zygotes for injection of transgenes. In such species, the DNA must be injected into the cytoplasm (Chourrout et al. 1986). Regardless of the species, the outcomes of the microinjection technique are reported to be low (Mann and Mcmahon 1993), as it involves multiple steps and demands advanced skills in various steps, including harvesting of the embryos and microinjection. Overall, the technology is time-consuming, tedious, and least consistent. Consequently, recent research efforts were largely focused on the development of alternative methodologies for enhancing the efficiency and minimizing the cost of producing the genetically-altered farm animals.

Alternative strategies that were investigated included: (1) injection of transgene along with a restriction enzyme into the zygote (Seo et al. 2000) which resulted in a twofold increase in the production of transgenic mouse founders; (2) microinjection of DNA coated with the bacterial recombinase (RecA), which also increased the overall efficiency for producing transgenic offspring (Maga et al. 2003) in goats and pigs and resulted in better embryo survival and transgene integration; and (3) more recently, combining microinjection with transposable elements or genome editors and cell-based transgenic technologies such as SCNT for the generation of transgenic animals.

13.2.1.2 Transposon-Mediated Transgenesis

Transposons have been successfully leveraged for transgenesis in flies, fish, frogs, mice, and rats (Muñoz-López and García-Pérez 2010). In mammals, the most commonly used transposons systems are the Sleeping Beauty (SB), *piggybac* (PB), and Tol2 systems, and the standard method of delivery is pronuclear microinjection into zygotes. Many of the drawbacks of classical transgenesis can be overcome by transposition-mediated gene delivery, which increases the efficiency of chromosomal integration and facilitates single-copy insertion events (Kues and Niemann 2011; Kumar et al. 2020), however still lacking strong target-site specificity. Combining transposon systems with pronuclear injection greatly increased the transgenic efficiency which in parallel to the viral transductions (see below), brings about several fold increase in the production efficiencies for gene modified animals in comparison to the traditional PNI techniques (Bosch et al. 2015). In mammalian species, it was successfully applied in mouse (Ding et al. 2005; Dupuy et al. 2002; Mátés et al. 2009), rat (Jang and Behringer 2007), rabbit (Katter et al. 2013), and pig (Carlson et al. 2011). The first transposon-mediated transgenic pigs have recently been generated (Kues et al. 2010; Garrels et al. 2011) by a microinjection technique (Iqbal et al. 2009), as well as by SCNT (Jakobsen et al. 2011). As mentioned earlier, pronuclei in pig and cattle embryos are obscured by lipid droplets which can be

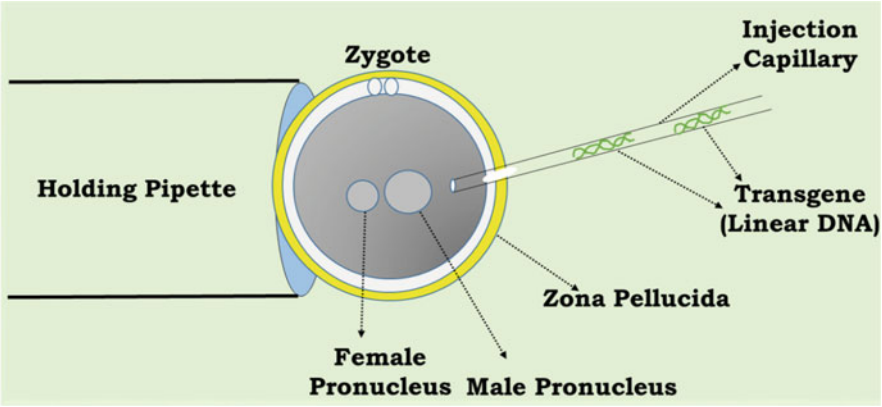

Fig. 13.5 Cytoplasmic DNA microinjection

overcome by injection of transposon and transposase combinations directly into the ooplasm/cytoplasm or into the zygote (Fig. 13.5). With the use of recently developed transposases, such as the PB system that is highly active, potent, and effective for successful transmission through cytoplasmic injection has facilitated production of gene modified rodents (Marh et al. 2012), pigs (Garrels et al. 2011; Li et al. 2014; Garrels et al. 2016a), cattle (Garrels et al. 2015, 2016b), and sheep (Bevacqua et al. 2017) at a higher efficiency.

13.2.1.3 Retro- and Lentiviral Methods

To increase the probability of integration, pseudotyped retroviruses which carry genetic material as RNA instead of DNA, which integrate into the host genome using a mechanism not by the way of homologous recombination (HR), and more importantly lack the ability to auto-replicate have been leveraged for generating transgenic animals. The first transgenic mice using this method were generated by Jaenisch and Mintz in the year 1974 (Jaenisch and Mintz 1974). They produced healthy mice by microinjecting SV40 DNA (carrying copies of foreign viral DNA) into zygotes. Offspring generated with this technique are usually mosaic, and the transmission of the transgene is feasible only if the virus integrates into the prospective germ cells. The mosaic founders can be backcrossed until homozygous transgenic animals are produced. Recent research efforts have revealed that lentiviruses can be leveraged to overcome the hurdles of classic viral methods by obtaining higher expression and integration levels (Niemann et al. 2005). Stable transgenic lines have been established by injecting the lentiviruses into the perivitelline space of cattle and porcine zygotes, which resulted in an enhanced proportion of transgenic calves/piglets, respectively (Hofmann et al. 2003; Whitelaw et al. 2004). Lentiviral mediated gene transfer in domestic animals has demonstrably higher efficiency of generating transgenic animals due to multiple integration events. Unfortunately, multiple integrations in the genome bring with it the undesirable consequences such as oncogene activation and/or insertional mutagenesis (Biasco et al. 2018).

Besides high incidence of mosaicism, gene silencing due to the presence of viral sequences are other additional problems identified with this approach (Park 2007).

13.2.1.4 Sperm-Mediated Gene Transfer (SMGT)

Sperm is a natural vector that delivers paternal DNA into the oocyte to give rise to a zygote. This natural ability of the sperm cells to bind and internalize exogenous DNA and to carry the "cargo" into the oocyte during fertilization for generating transgenic animals was first demonstrated in rabbits by Brackett et al., in the year 1971 (Figs. 13.6 (Brackett et al. 1971), and soon after in mice (Lavitrano et al. 1989). The SMGT technique was also applied across various other species, including chicken (Nakanishi and Iritani 1993), pig (Lavitrano et al. 1989, 2005), goat (Zhao et al. 2009), and cattle (Perez et al. 1991), with varying success. SMGT proved to be cost effective and appeared to be more efficient and has the potential to be applied for large-scale applications. However, difficulties in reproducibility and low efficiency due to low uptake of exogenous DNA by the spermatozoa, lowered potential of transfected spermatozoa for fertilizing the oocytes, and a measurable failure in delivering the transgenes into the animal genome, have not surprisingly resulted in inconsistent results and considerable controversy for several years (Spadafora 2008; Gandolfi 2000). To enhance the effectiveness of sperm uptake of DNA, many other approaches like attaching the recombinant DNA to the sperm head via an antibody amalgamated to DNA (Chang et al. 2002), lipofection (Lai et al. 2006) or electroporation (Rieth et al. 2000) methods have also been tested by placing the DNA inside the sperm head. In order to overcome the hurdles in the way of delivery of exogenous DNA by sperm, another technique called intracytoplasmic sperm injection (ICSI) has been tested, aptly named ICSI mediated Transgenesis (ICSI-Tr). In this method, frozen-thawed spermatozoa is coupled with exogenous DNA and is introduced into the cytoplasm of oocytes (Fig. 13.7) for the production of a transgenic animal (Moisyadi et al. 2009) as was first demonstrated in mice (Perry et al. 1999). In species other than mice in which the procedure was attempted resulted in

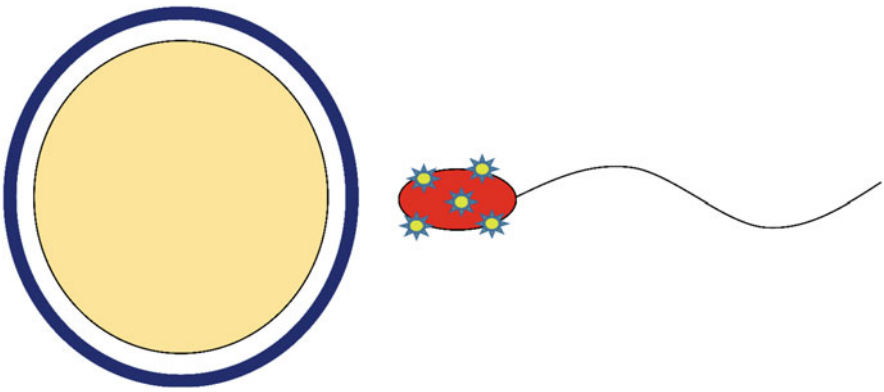

Fig. 13.6 Sperm-mediated gene transfer

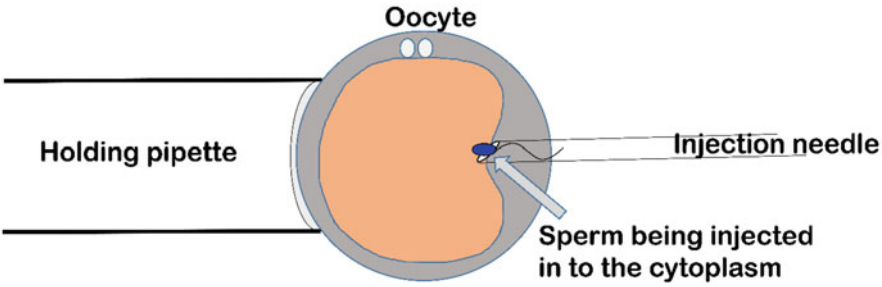

Fig. 13.7 Intracytoplasmic sperm injection technique

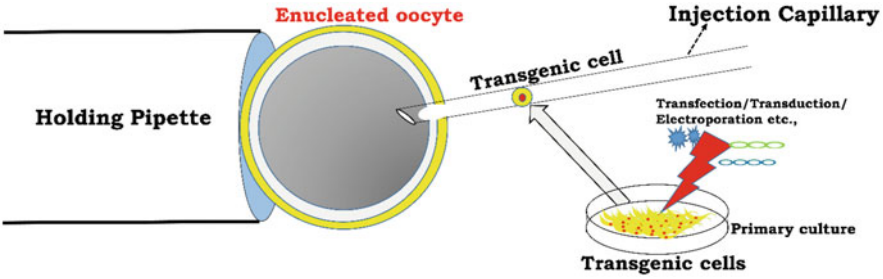

Fig. 13.8 Embryonic stem cell-mediated transgenesis technique

markedly lower efficiency. To enhance the efficiency of ICSI-Tr, other strategies were employed including integration of ICSI-Tr with a recombinase, integrase, and transposase to actively assist the integration of transgenes into the host genome, which has fostered transgenesis rates several fold higher over the unassisted ICSI-Tr in mice (Shinohara et al. 2007).

13.2.2 Cell-Mediated Approaches

13.2.2.1 Embryonic Stem Cell (ESC)-Mediated Gene Transfer

In the past three decades since their initial discovery, ESC have been used extensively to generate transgenic animals with site-specific modifications. In this method, the ESC are isolated from the inner cell mass (ICM) of donor blastocysts, cultured in vitro, transfected with the appropriate transgene construct by a suitable transfection technique (Fig. 13.8), and selected for stable integration using selectable marker. The selected ES-cells are microinjected into blastocysts for eventual contribution to somatic and germ line lineages in the resulting chimeric animal. Stem cells being the derivatives of undifferentiated embryonic cells can contribute to any cell type and hence can potentially contribute to the entire organism. Using this methodology, targeted and defined mutations can be precisely engineered via homologous recombination. But the lack of availability of analogous and true ESC in livestock

species is the major hurdle in capitalizing on this technique (Malaver-Ortega et al. 2012). The availability of induced pluripotent stem cells (iPSC) that have the same characteristics as that of ESC provided an alternative to the ESCs, with the theoretical possibility of generating them from any individual species (Talluri et al. 2021; Dharmendra Kumar et al. 2021; Kumar et al. 2015; Anand et al. 2016). In fact, iPSC derived germline competent offspring were produced in mouse and rat (Hamanaka et al. 2011; Okita et al. 2007). In contrast, similar trials with livestock iPSCs were proven be less successful with only one study reported in pigs (West et al. 2010). For producing the transgenic animals using the iPSC, a thorough understanding of the derivation of iPSCs, their culture conditions, and maintenance of pluripotency is needed (Dharmendra Kumar et al. 2021). Recent advances in iPSC derivation methods offer hope for this effort (Soto and Ross 2016; Scarfone et al. 2020).

13.2.2.2 Somatic Cell Nuclear Transfer (SCNT)

This technique has been the "go-to" method for generating transgenic farm animals with desired site-specific alterations (West and Gill 2016). However, SCNT is not a trivial exercise. It involves many important stages, with each stage critically impacting the efficiency of cloning. These include:

1. genetic modification and culture of donor cells.
2. enucleation.
3. introducing the donor cell or nuclei adjacent to the enucleated oocyte.
4. fusing the donor cells with the oocyte either via an electric pulse or insertion directly into the cytoplasm.
5. activation of the reconstructed embryos, and
6. in vitro culture of generated embryos and transfer into synchronized surrogate recipients (Polejaeva 2021) (Fig. 13.9).

Earlier, lipofection and electroporation techniques were used for delivering the constructs into the cells (Kumar et al. 2020). Recently, many other robust methods like transposon-mediated transgenesis (Kues and Niemann 2011), viral-mediated delivery methods (pseudotyped lentivirus or recombinant adeno-associated virus (rAAV)), and electroporation have been developed. Till date many transgenic farm

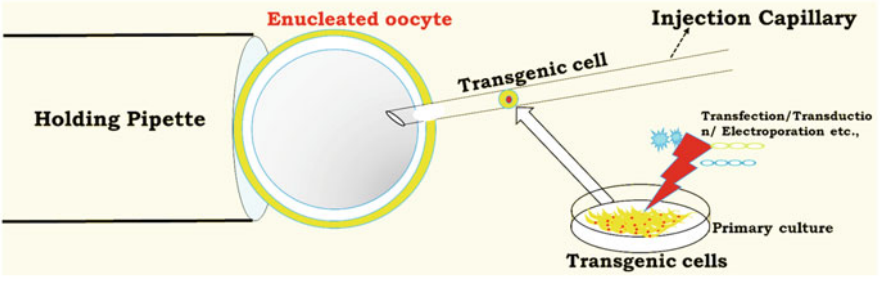

Fig. 13.9 Somatic cell nuclear transfer technique

animals carrying a variety of genetic modifications have been generated via SCNT. In spite of this great success, still some hurdles do exist for the practical application of SCNT, which include: (a) extremely low cloning efficiencies in essentially all species; (b) frequent abnormalities in the extraembryonic tissues, such as placenta of the cloned animals (Ogura et al. 2013); and (c) abnormalities in produced clones, including respiratory defects, immunodeficiency, obesity, and untimely mortalities (Ogura et al. 2013; Loi et al. 2016). These findings highlight the current limitations that hinder the normal development of cloned embryos and animals (Matoba and Zhang 2018).

13.3 Novel Approaches for Genome Modification

The introduction and successful validation of genome editing (GE) technologies have been a game changer for transgenesis in livestock species, which has not witnessed a major breakthrough since the advent of SCNT. In livestock species, the designer site-specific nucleases such as ZFN, TALEN, and CRISPR/Cas may be delivered by either microinjecting into the cytoplasm of zygotes, or transfecting the donor cells that serve as donors for SCNT (Fig. 13.10). A series of customized endonucleases have been developed that have the capacity to specifically target the genomic region of interest and introduce DSBs (Urnov et al. 2005; Miller et al. 2007, 2010; Christian et al. 2010; Kim and Kim 2014). DNA-based nucleases such as meganucleases, ZFNs, TALENs, and RNA-based nucleases such as CRISPR/Cas can recognize specific sequences and introduce a DSB at the target site, triggering endogenous repair at the cut-site (Table 13.3). The DSB can be repaired either through an error prone NHEJ or HDR pathways (Ceccaldi et al. 2016; Li et al. 2017; Lee et al. 2018). The NHEJ pathway results in the introduction of short indels at the site of strand break, while HDR utilizes a template DNA to knock-in the

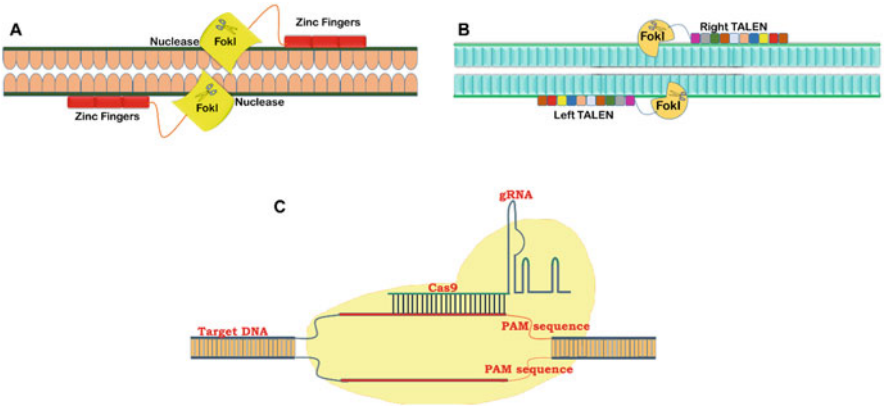

Fig. 13.10 Mechanisms of emerging genome editing strategies A. Zinc Finger Nucleases. B. TALENs C. CRISPR/CAS9 technology

Table 13.3 Characteristic features of genome editing tools, viz. ZFN, TALEN, and CRISPR/CAS

Characteristic variable	ZFN	TALEN	CRISPR
Backbone origin	Eukaryotes	Plant pathogen Xanthomonas *spp* (bacteria)	Prokaryotes S. *pyogenes* (bacteria)
Specificity module	ZFP	TALE	sgRNA (crRNA + tracr RNA complex)
Mode of DNA recognition	Protein-DNA	Protein-DNA	RNA-DNA-protein
Length of target site (bp)	18–36	30–40	20 bp + PAM (NGG) sequence
Rate of mutation (%)	10	20	20
Endonuclease/ cleavage module	FokI	FokI	Cas9
Binding specificity	Trinucleotide	Single nucleotide	1:1 nucleotide paring
Working mechanism	DNA/protein recognition, DSB and its repair pathway	DNA/protein recognition, DSB and its repair pathway	DNA/RNA recognition, DSB and its repair pathway
Reprogramming efficiency	Relatively low	Relatively low	Relatively high
Off-targeting	High	Low	Variable
Ease of designing	Difficult	Feasible but labor intensive	Easy
Ease of delivery	Easy	Difficult	Moderate
High throughput targeting	No	Limited	Yes
Multiplexing	Challenging	Challenging	Feasible
Biased repairing pathway	NHEJ	HDR	NHEJ
Immunogenicity	Less	Unknown	Unknown
Cytotoxicity	Variable to high	Low	Low
Vector packaging and therapeutic delivery	Difficult	Difficult	Easy
Economical in designing and cost effective	No	Moderate	Yes

transgene or sequence replacement. The editors are capable of improving the targeting efficiency up to 100,000, and gene disruption via mutagenic DNA repair is stimulated at a similar frequency (Moehle et al. 2007). Within a relatively brief time from when they are first introduced, several researchers have efficiently applied these editing tools and succeeded in producing the GE pigs, sheep, goats, and cattle covering a wide range of potentially beneficial genetic modifications, both for

Table. 13.4 Genome edited farm animals produced either through SCNT or microinjection and using repairing mechanisms, viz. HDR and NHEJ

Genome editing tool	Species	Method of transgenesis	Repairing pathway	Targeted/ altered gene	Reference
ZFN	PIG	SCNT	NHEJ	PPAR	Yang et al. (2011)
				α 1, 3GT	Hauschild et al. (2011)
				eGFP	Watanabe et al. (2010), Whyte et al. (2011),
		CMI	HDR	ReIA	Lillico et al. (2013, 2016)
	CATTLE	SCNT	NHEJ	BLG	Yu et al. (2011)
				GDF8	Luo et al. (2014)
TALEN	PIG	SCNT		LDLR	Carlson et al. (2012)
		CMI		B2M	Wang et al. (2016)
	CATTLE	CMI		GDF8	Proudfoot et al. (2015)
		SCNT	HDR	Polled locus	Carlson et al. (2016)
		SCNT		SP110	Wu et al. (2015)
	SHEEP	CMI	NHEJ	GDF8	Proudfoot et al. (2015)
CRISP/ CAS9	PIG	SCNT		SLA-1,2,3	Reyes et al. (2014)
				α 1,3GT, CMAH, B4GalNT2	Butler et al. (2016)
		CMI		CD163	Whitworth et al. (2016), Burkard et al. (2017)
				vWF	Hai et al. (2014)
				α 1,3GT	Petersen et al. (2016)
	CATTLE	SCNT	HDR	NRAMP1	Gao et al. (2017)
		CMI	NHEJ	PRPN	Bevacqua et al. (2016)
	GOAT	SCNT	NHEJ	GDF8	Ni et al. (2014)
		CMI		GDF8. FGF	Wang et al. (2015)

agricultural and biomedical applications (Table. 13.4) (Kalds et al. 2019; West and Gill 2016; Perisse et al. 2020). The relative ease with which the nucleases can be assembled and delivered, and their relatively high efficiency in introducing targeted genetic modifications has proven to be a game changer in this field, as evidenced by the deluge of publications in the past few years. These results highlight successful employment of nucleases in a wide variety of species rendering them beneficial for understanding the complicated biological systems, creating specific cell lines, producing transgenic animals, generating large animal models for human diseases, and even for treating genetic diseases in human. Below is a brief description of the nucleases and their application towards genetic modification in animals.

13.3.1 Meganucleases

Meganucleases are the first-generation site-specific nucleases for introducing targeted double strand breaks in the genome (Daboussi et al. 2014). However, the meganucleases did not find wide adaptability, and the literature pertaining to their use in livestock is minimal (Bevacqua et al. 2013; Wang et al. 2014).

13.3.2 Zinc Finger Nucleases (ZFNs)

The first use of ZFN was reported by Kim et al. (Kim et al. 1996) approximately 15 years after the discovery of zinc finger (ZF) domain transcription factors. The ZF contains amino acids (approx. 30) that form two anti-parallel ß-sheets opposing an α-helix (Pabo et al. 2001). The ZFNs are chimeric proteins composed of Cys_2-His_2 DNA-binding domain with a zinc ion. Trimeric (or more) zinc finger domains are usually fused to one half of FokI endonuclease (Urnov et al. 2005). The FokI domains must dimerize to reconstitute the holoenzyme for inducing the DSB. Fusion of monomeric FokI domains therefore requires binding of two ZFN molecules on opposite strands in a tail- to-tail fashion and segregated by 6–8 base pairs (bp), with dsDNA break occurring in the spacer region (Smith et al. 1999). This coupled with the ability to custom build ZFs to bind to almost any triplet base and multiple ZFs combining to form larger DNA recognition domains, further enhances the efficacy, range, and specificity of GE in any given cell type (Petersen and Niemann 2015). For genome modification, a plasmid DNA or mRNA encoding a particular ZFN can be instituted either into the embryos through microinjection, or into the cells via transfection (Hauschild-Quintern et al. 2013). ZFN pair binds to the specific target after translation and the FokI domain will dimerize to cleave the DNA. Among livestock, ZFNs were first experimentally validated, and their efficacy was proven in pigs (Yang et al. 2011; Whyte et al. 2011) through knocking down the eGFP and PRARγ genes.

13.3.3 Transcription Activator-like Effector Nucleases (TALENS)

Transcription activator-like effector nucleases (TALENs) share similarities with ZFNs and comprise a non-specific FokI nuclease domain fused to a customizable DNA-binding domain (Joung and Sander 2013). This domain is composed of highly conserved repeats derived from transcription activator-like effectors (TALEs), which are proteins secreted by *Xanthomonas* bacteria for altering the transcription of genes in host plant cells (Boch et al. 2009). The TALE central repeat domain consists of 33–35 amino acids of repeating units (Deng et al. 2012). Each repeat is mostly identical except for two highly variable amino acids at positions 12 and 13, referred to as the repeat variable di-residues (RVDs) that are hypervariable and which determine the TALENs' specificity (Deng et al. 2012; Mak et al. 2013). TALENs have replaced the ZFNs very rapidly and proved to be an alternative to ZFNs for GE

(Fernández et al. 2017). Although TALENs utilize TALE motifs as the DNA recognition modules and ZFNs use the ZFs as the DNA-binding modules, both use the *Fok*I catalytic domain as the DNA cleavage module (Petersen 2017). However, unlike ZFNs, no re-engineering of the linkage between repeats is necessary to construct long arrays of TALEs, and with the theoretical ability of targeting any specific sites in the genome (Gaj et al. 2012). Though both ZFNs and TALENs have equal cleavage efficiencies, TALENs have demonstrated limited toxicity to the cells in comparison to ZFNs when targeting the same locus (Chandrasekaran et al. 2017). TALENs were also employed for generating knockout zebrafish and rats (Tesson et al. 2011; Sander et al. 2011; Huang et al. 2011), cattle, sheep (Proudfoot et al. 2015), and pigs (Carlson et al. 2012), thus demonstrating versatility for GE in a wide range of species (Carlson et al. 2011; Proudfoot et al. 2015).

13.3.4 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)

The CRISPR/Cas is an adaptive immune system found in many bacterial and archaea species against viral and other exogenic agents (Jinek et al. 2012). Among the genome editors, the CRISPRs have emerged as a clear winner because of their simplicity in design and assembly, precision at the target site, and high efficiency in introducing DSBs (Li et al. 2020; Wada et al. 2020). Specifically, the *Streptococcus pyogenes* type II CRISPR/Cas system is the most widely used CRISPR system (Adli 2018). This system uses small noncoding RNAs (short guide RNA (sgRNA)) to target a Cas nuclease to a matching 20 nucleotide protospacer sequencing abutting a 3 bp protospacer adjacent motif (PAM) sequence on the opposite strand (Fu et al. 2014). A major limitation of CRISPRs is their propensity to cleave at extraneous non-targeted sites (Fu et al. 2014). Though off-targeting concerns can be limited by careful sgRNA design (Akcakaya et al. 2018), it is not always possible when the target region is within a small window. Finding good target sites can be a challenge when the intended site is located in AT-rich regions of the genome, which is further complicated by the requirement for a PAM sequence adjoining the CRISPR site. That said, Cas nucleases are being engineered and/or novel variants identified with promiscuity to alternative PAM sites (Leenay and Beisel 2017), thereby greatly extending the targeting range of this system. Several new CRISPR systems have been discovered, extending the toolbox for efficient and precise targeting and/or manipulation (Komor et al. 2016). These systems include catalytically inactive/dead Cas9 (dCas9) fused with transcriptional activator or repressor domains for gene regulation, epigenetic modifiers (epigenetic modification), chromatin regulators, and base editors (Adli 2018). Single-nucleotide alterations have also been engineered using base editor systems fused to dCas9, and further versions have been developed using Cas9 nickase (nCas9) (Komor et al. 2016; Eid et al. 2018). These enable new and more precise forms of genomic modifications. The CRISPR/Cas methodology was first successfully adopted for use in mammalian genomes in 2013 (Cong et al. 2013; Mali et al. 2013). During the same year, mutant mice with multiplex genetic

modifications have been created, paving the way for a multitude of similar efforts in almost all economically important livestock species (Wang et al. 2014; Shen et al. 2014; Yang et al. 2018) (Table 13.5). Due to their simplicity, affordability, and customizability, CRISPR systems have unleashed a great biotechnological revolution in basic research, biomedicine, and in agriculture.

Table 13.5 Transgenic animals produced using CRIPSR/Cas9 technology

Species	Method of gene transfer	Targeted Gene	Reference	Aim/Outcome of the application
Pig	Somatic cell nuclear transfer	α1,3GT, CMAH, B4GalNT2	Butler et al. (2016)	Multiple gene knockouts for xenotransplantation studies
	Cytoplasmic DNA injection	CD163	Whitworth et al. (2016)	Resistance against PRRSV
	Somatic cell nuclear transfer	MSTN	Bi et al. (2016)	Generation of isozygous, MSTN knockout cloned pigs
	Cytoplasmic DNA injection	NANOS2	Park et al. (2017)	Generation of genetically superior male
	Cytoplasmic DNA injection	CD163 SCRC5	Burkard et al. (2017)	PRRSV
Sheep	Cytoplasmic DNA injection	MSTN	Han et al. (2014), Crispo et al. (2015)	Enhanced muscle differentiation and growth
	Cytoplasmic DNA injection	ASIP	Zhang et al. (2017)	Sheep coat color Pattern alteration
	Cytoplasmic DNA injection	BMPR-IB	Zhang et al. (2017)	Increased litter size due to increased ovulation rate
	Cytoplasmic DNA injection	FGF5	Li et al. (2017)	Increased yield and quality of the wool
Goat	Cytoplasmic DNA injection	MSTN And FGF5	Wang et al. (2016)	Fiber length enhancement
	Cytoplasmic DNA injection	GDF9	Niu et al. (2018)	Introduction of point mutations
	Somatic cell nuclear transfer	BLG	Zhou et al. (2017)	Production of beta-lactoglobulin knockout goats
Cattle	Somatic cell nuclear transfer	POLLED	Carlson et al. (2016)	Polled cattle generation
	Somatic cell nuclear transfer	NRAMP1	Gao et al. (2017)	TB resistant cattle
	Somatic cell nuclear transfer	IARS	Ikeda et al. (2017)	Repair of the IARS mutation

13.4 Applications and Use of Transgenic Animals

Since the dawn of domestication, the livestock were selected for desirable traits (Murray and Anderson 2000). These methods though successful have proven to be slow in attaining desirable outcomes. With the availability of advanced technologies like transgenesis, it is now possible to alter genomes and introduce exogenous traits via precise genetic modification and accordingly confer the desired benefit (Hansen 2000; Niemann et al. 2009; Niemann and Kues 2007; Wheeler et al. 1991; Wheeler 2007). Practical applications of transgenesis in livestock production include enhanced quantity and quality of milk and its composition, more efficient utilization of feed, enhanced growth rate, leaner meat, increased immunity against the infections, enhanced reproductive performance and prolificacy, resistance to various dangerous diseases, and for producing various effective drugs and modified cell and tissue lines for the study of effectiveness of these drugs used for biomedical applications (Wheeler et al. 1991; Ward and Nancarrow 1995). There are a wide range of transgenic animals that are already being produced (poultry, pigs, goats, sheep, and cows), with desirable traits (Table. 13.6). Genome edited and model organism developed using this technology are proving to be indispensable for increasing our comprehension of disease etiology, functionality of various genes, etc., (Jaenisch and Mintz 1974; Gordon 1989). Few representative major themes are discussed below.

13.4.1 Preclinical Models

In the initial phases of transgenesis, research was focussed on developing lab animal (mice, rodents) models for investigating gene function or as preclinical models mimicking human diseases. The rodent models have several advantages including the ease of handling and low generation intervals (Hamernik 2019). However, there is a growing consensus among the biomedical scientists that the rodent models cannot fulfil all the requirements of research, for example, in cases where the size of the animal, long lifespan and similarity in organ size, anatomy, and physiology are paramount. Thus, the focus is now being diverted for the generation of custom preclinical transgenic farm animal models which mimic the human disease conditions more accurately, aid in the development of therapies (Zhao et al. 2019).

13.4.2 Improving Reproductive Performance and Fecundity

Enhancing the fertility and reproductive efficiency are vital considerations for animal agriculture. To enhance the reproductive efficiency in farm animals, several candidate markers were identified. Few of them are highlighted here:

- mutations in oocyte-derived growth factors of the TGF-beta family including growth differentiation factor 9, bone morphogenetic protein 15, and activin

Table 13.6 List of transgenic food animals for agricultural applications

Species	Transgene	Targeted trait	Year
Pig	Phytase	Feed uptake; decreased phosphorus in manure	2001
	Growth hormone, growth hormone releasing factor, insulin-like growth factor-1	Growth rate	1990
	cSKI	Muscle development	1992
	Omega-3 (Fat-1)	Meat composition	2011
	Lysozyme	Piglet survival	2006
Goat	Stearoyl-CoA desaturase	Milk composition	2004
	Lysozyme	Animal health	2006
	Prion protein (PrP) shRNA	Animal health	2006
	Lactoferrin	Prophylactic treatment	2008
	Human beta-defensin 3	Milk composition	2013
	Myostatin shRNA	Increased muscle yield	2013
Sheep	Growth hormone, growth hormone releasing factor,	Growth rate	1998
	Prion protein (PrP)	Animal health	2001
	Omega-3 (Fat-1)	Meat composition	2013
Cattle	Lysozyme, Lactoferrin	Milk composition; animal health; mastitis resistance	2002, 2011
	α−,κ-casein	Milk composition	2003
	Lysostaphin	Mastitis resistance	2005
	Prion protein (PrP)	Animal health	2007
	β-Casein miRNA	Milk composition	2012
	Omega-3 (Fat-1)	Milk composition	2012
	SP110	Bovine tuberculosis resistance	2015

receptor-like kinase 6, which are promising candidates for increasing ovulation rates and thus litter size (Mcnatty et al. 2005).

- Booroola fecundity (FECB) and estrogen receptor (ESR) are also other promising targets.
- Mutations in the *BMPR-IB (FecB)* gene in sheep (Fabre et al. 2006) and ESR gene in pigs (Rothschild et al. 1996) have been shown to increase the number of ovulations and subsequently the litter size (Fabre et al. 2006; Rothschild et al. 1996; Yalew et al. 2020).
- The same result was also obtained in many of the sheep breeds with incorporation of *FECB* increasing prolificacy in sheep (Gottlieb and Wheeler 2008; Piper et al. 1985).
- A novel gene (which makes the posteriors of the animas red when in estrus) from baboons was introduced into pigs to enhance the visibility of estrus symptoms (Seidel Jr et al. 1999), thereby potentially contributing to increased fertility (Saniotis 2007).

13.4.3 Replacement of Blood and Blood Constituents

Donated blood remains the major source of essential and important components of the blood. This use of donated blood as the major source of blood is limiting due to a lack of qualified blood donors, disease concerns, among others. Transgenic pigs that produce functional human hemoglobin (HHb) have been produced (Whyte et al. 2011; Yang et al. 2021). A large pig can donate 9–10 liter of blood over the course of a year without detrimental health effects, thereby yielding 500–1000 g of purified HHb. This purified protein showed oxygen binding characteristics comparable to HHb; however, the proportion of red blood cells containing human form of Hb is low (Kues and Niemann 2011). Other strategies include production of human blood substitutes by crosslinking the superoxide-dismutase system to Hb chemically (Niemann and Petersen 2016).

Human serum albumin is in a huge demand as a therapeutic for various clinical complications including liver disease, traumatic shock, etc. (Peng et al. 2015). To circumvent this shortfall, cDNA of human albumin was incorporated into the endogenous locus of the pig albumin to generate transgenic pigs (Peng et al. 2015). As the donated human blood remains limiting, alternative strategies such as the production of HSA in transgenic pigs remain a viable option. Transgenic animals carrying human antibody genes can be leveraged for production of human polyclonal antibodies for treatment of various diseases or other indications like organ transplant rejection, cancer, and autoimmune diseases (Brüggemann et al. 2015).

13.4.4 Improving Milk Production and Altering Composition of Milk

With the advent of genetic engineering, it is now possible to increase the milk production and efficiency, and/or alter the composition of milk produced from the farm animals. These technologies have provided novel avenues and significantly propelled the efficiency of gene pharming by generating the animals that produce useful recombinant proteins in milk. Targeting the mammary gland for the synthesis of proteins is more advantageous, because of the mammary epithelial cells' ability to append appropriate post-translational modifications and secrete biologically active proteins in abundance. Previously transgenic mice, rabbits, pigs, goats, and sheep have been generated in which the biologically active proteins of therapeutic value were secreted in milk (Simons et al. 1987; Bühler et al. 1990; Ebert et al. 1991; Wall et al. 1991; Wright et al. 1991). Important other proteins like human blood clotting factor IX for treating hemophilia, alpha-1 antitrypsin used for treating emphysema, protein C required for blood clotting, and HSA were directed for production in milk of transgenic cows, pigs, sheep, and goats (Moura et al. 2011; Velander et al. 1992). Transgenic pigs with enhanced milk production and increased concentration of lactose in their milk were produced by altering the lactalbumin gene in the mammary gland, which resulted in the increase in the growth and survival of piglets (Stinnakre et al. 1994).

The ratios of lipids and proteins present in the milk differ between various species and between different breeds within the species (Gibson 1991). A higher ratio between lipids and proteins in milk is most desirable, and this can be achieved by increasing expression of important milk proteins (like casein), or altering the genes expressed in the lipid biosynthesis pathway or lipid metabolism in the mammary gland. Through this strategy, the relative expression of beta- and kappa casein in the mammary gland can also be increased resulting in higher yield of curd and cheese (Kues and Niemann 2004) and increasing the value of the milk (Maga et al. 2003, 2006a, b; Sun et al. 2018; Fahrenkrug et al. 2010; Eenennaam et al. n.d.). Likewise, a major whey protein, β-Lactoglobulin (BLG) which is present in cow and goat milk but not present in human milk elicits mild to serious allergic reactions (Villa et al. 2018; Layman et al. 2018) and has been eliminated by the use of ZFN and TALENs (Yu et al. 2011; Wei et al. 2018). With the employment of precise and robust GE technologies, we can anticipate production of nutraceuticals from farm animals in the coming years.

13.4.5 Disease Resistance

Various diseases caused by bacteria, virus, or other pathogens will not only hamper the health and welfare of the animals, but also limit productivity from these animals. However, resilience to these diseases can be augmented by manipulating host factors regulating immunity (Islam et al. 2020). Recognition of the polymorphic and monomorphic genes which are part of major histocompatibility complex (MHC) and which can effectively influence the host immune response are attractive candidates for GE and for conferring resistance to a disease (Benacerraf 1985; Germain 2011; Menchaca 2021). There are many aspects of disease resistance or susceptibility in livestock that are genetically determined and could be targeted. Representative examples are below:

- Cattle lacking prion protein have been generated which prevents infection and spread of bovine spongiform encephalopathy (BSE) (Richt et al. 2007a, b). The same modifications were also incorporated in other species like ovine (Denning et al. 2001), caprine (Yu et al. 2006, 2009; Benestad et al. 2012), and cattle (Kuroiwa et al. 2004) that can withstand to scrapie and BSE, respectively.
- Lysostaphin transgenic cows were shown to be resistant to mastitis caused by *staphylococcus* (Wall et al. 2005; Schmelcher et al. 2012). The milk of these cows contains lysostaphin which has the ability to counter mastitis causing *staphylococcus* in a dose-dependent manner (Kerr et al. 2001).
- Classical swine fever (CSF) virus is a major threat to the pig industry, which causes huge losses to the pork industry (Xie et al. 2018). CSF virus resistant pigs were generated by specifically incorporating small RNAs targeting CSF virus into the *ROSA*26 locus (Xie et al. 2017).

- In an effort to enhance the immunity in mice, pigs, and sheep, and counter the infections, immunoglobulin- A (IgA) gene has been altered transgenically (Lo et al. 1991).
- Transgenic sheep resilient to the *Visna virus* infection were generated by sheep transgenic for *Visna virus* envelope protein (Clements et al. 1994).
- Transgenic pigs and birds were produced which are completely free from influenza infections (Lyall et al. 2011) by introducing synthetic short hairpin RNA mimicking the influenza A virus.
- Pigs lacking *CD163* gene were generated and were shown to be resilient to PRRSV infection, with no systemic viremia observed in $CD163^{-/-}$ pigs following viral challenge (Whitworth et al. 2016).
- African swine fever (ASF) is a highly infectious and deadly disease of pigs (Tait-Burkard et al. 2018). It has been hypothesized that introducing the variant of *RELA* found in warthogs (Palgrave et al. 2011) which are reservoirs of the disease, to domestic pigs will confer resistance to the disease. Using a ZFN, researchers were able to convert the pig *RELA* to mimic warthog (Lillico et al. 2016); however, data to show resilience to ASF virus have yet to be reported.
- *Mannheimia* (*Pasteurella*) *haemolytica* infection causes epizootic pneumonia (shipping fever), enzootic pneumonia, and peritonitis in calves, lambs, and sheep. ZFNs have been used to introduce a single amino acid change in the cattle CD18 protein, and the leukocytes from resultant fetuses were shown to be resistant to *M. haemolytica* leukotoxin-induced cytotoxicity (Shanthalingam et al. 2016).
- Bovine tuberculosis (bTb) is a potential zoonotic that has a huge negative impact on productivity in cattle and buffalo and is becoming a serious threat (Grange 2001). Polymorphisms in the natural resistance-associated macrophage protein 1 (*NRAMP1)* gene are correlated with resilience to bovine tuberculosis in cattle (Cheng et al. 2015), and cattle were made transgenic using CRISPR/Cas9 (Gao et al. 2017). Additional candidates that could be employed are *SP110* (SP110 Nuclear Body Protein) gene which has been evolved as a potential candidate for controlling the infection by *M. bovis* and its multiplication (Zhao et al. 2019).
- Pigs resistant to foot and mouth disease virus (FMDV) were generated using shRNAs targeting the FMDV (Hu et al. 2015).

13.4.6 Xenotransplantation

Xenotransplantation is the concept of replacing an organ from suitable species into other species (Niemann and Petersen 2016). To mitigate the shortfall in the availability of suitable organs for transplantation in humans, transgenic pigs with improved compatibility for transplantation into humans, and with the anatomy and physiology closely matching to that of humans have been the subject of decades long investigation (Kues and Niemann 2011; Kumar et al. 2015, 2020; Niemann et al. 2005; Dharmendra Kumar et al. 2021; Kues and Niemann 2004; Platt and Lin 1998). The use of xenografts from genome edited pigs, together with more sophisticated

immunosuppressive treatments, may prove successful for this effort (Sykes and Sachs 2019). One important hinderance to this process is the hyperacute immune reaction which further complicates tissue rejection (Kummer et al. 2020). Additionally complications arise from cellular rejection (T-, B-, and NK cells). Therefore, multiple modifications are envisaged and constantly investigated to "humanize" the pig organs for transplantation. Genome editors have improved the efficiency of introducing desired edits and for development of genetically modified pigs compatible for xenotransplantation (Hauschild et al. 2011; Miyagawa et al. 2015; Nagashima and Matsunari 2016). A by-product of this effort is the generation of hypoallergenic galactosyl transferase knockout "Gal-safe" pigs that are approved by FDA for human consumption (Whitworth et al. 2016; Rao et al. 2016; Dolgin 2021). Furthermore, pigs with a genetic knockout of the B2M have been produced using CRISPR/Cas (Reyes et al. 2014; Wang et al. 2016), making them a putative universal organ donor for xenotransplantation. Moreover, the endogenous PERV sequences present in the porcine genome in multiple copies (~62 copies) have been completely knocked out by transfecting a porcine cell line with specific CRISPR/Cas vectors (Yang et al. 2015), highlighting the major gains from the use of these technologies.

13.5 Conclusion

In summary, advances in GE tools and associated transgenic technologies have facilitated efficient production of transgenic livestock carrying precise genetic modifications, which offers a great promise for accelerating genetic improvements in farm animals, and for their varied biomedical applications. Recent improvements in sequencing platforms add to our ability to investigate, enhance, or introduce preferable and advantageous traits into farm animals such as resilience to diseases and climate change, improved reproductive performance, augmenting the quality and quantity of agricultural products, to name a few. The emerging and evolving GE technologies are expected to empower the scientists to enact precision livestock breeding, and potentially ushering in a new era of "*breeding by design*."

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Offspring Sex Preselection in Mammals: An Update

14

Pradeep Nag, Shivanagouda Patil, A. Kumaresan, John Peter Ebenezer Samuel King, A. Manimaran, S. Jeyakumar, K. P. Ramesha, and D. Rajendran

Abstract

Producing young ones of desired sex has always been the desire of livestock farmers. Several attempts have been made to develop a method that efficiently skew the sex ratio of offspring towards females including separation bovine semen into fractions containing higher concentrations of X- or Y-bearing sperm and modulation of female genital micro-environment. Out of the several methods, sperm sex sorting by flow cytometry is the only viable technique available for use on commercial scale. Several countries are already using sexed semen in bovines and producing desired sex offspring depending upon the need. However, few reports indicate altered sperm quality and low conception rate with the use of sexed semen, probably due to the dye, sorting speed, pressure, laser light, electrical charging and deviation and changes in the medium that spermatozoa encounter during sorting. Development of the instrument for increasing the sorting rate and also purity of sorting without affecting the sperm viability and fertility is still an active area of research. On the other hand, evidences indicate the role of females in selection of sperm that is going to father her offspring. In this line, few studies indicate the female choice for sperm quality (and possibly the

P. Nag · S. Patil · A. Kumaresan (✉) · J. P. Ebenezer Samuel King
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India
e-mail: A.Kumaresan@icar.gov.in

A. Manimaran · S. Jeyakumar · K. P. Ramesha
Dairy Production Section, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

D. Rajendran
National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka, India

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sex of spermatozoa) and it has been reported that modulation of female genital micro-environment before insemination could be an option to skew the sex ratio.

Keywords

Skewing sex ratio · Sperm sexing · Flow cytometry · Oviduct · Micro-environment

14.1 Introduction

Sex ratio is defined as the ratio of males to females in a given population. According to Fisher's principle, sex ratio among the sexually reproducing species tends to unity to maintain the ecological balance. However, in commercial livestock production, especially in dairying, the utility of male animal is reducing with the time due to introduction of mechanization in agriculture and increased expenditure on rearing a male calf with very low or no output (Seidel Jr and Garner 2002). To increase the dairy industry's profitability and minimize the production of unwanted surplus male calves, it is desired to have more females that the males. Skewing sex ratio towards female is in greater requirement in countries like India where cattle slaughter is banned. These reasons created a need to identify methods for preselection of the offspring sex which could increase the profitability in dairy industry and also to meet the growing demands of milk and its products worldwide (Johnson 2000).

Sex preselection is required for rapid herd expansion and to practice aggressive culling of unproductive animals. Many approaches have been tried to pre-select the sex of an offspring or skew the sex ratio towards a particular sex. Basically, there are three approaches that could skew the sex of offspring towards the desired sex. First is to ensure the fertilization of oocyte by either X or Y sperm. Different techniques have been proposed in this area including albumin gradient centrifugation, swim up, electrophoretic separation and flow cytometry-based separation. Among these techniques, flow cytometry-based separation is the most successful technique till date. Secondly, predetermination of the sex of the embryo before transferring into the recipient. Embryonic sexing could be performed by karyotyping, H-Y antigen detection, determination of X linked enzyme, polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). Thirdly, modifying the maternal biochemical environment using different diets such as calcium, magnesium, glucose and fatty acids.

Earlier it was thought that the sex of an embryo was decided by the males as they produce both X and Y chromosomes while female produces only X chromosome bearing gametes. This led to the development of various methods to separate X and Y spermatozoa (sexed semen) based on density gradient centrifugation. The goal of sexed semen is to produce a sex-preselected calf; a new technique which has ventured into the prospects of finding replacements for a herd at a cheaper rate in dairy industry. This emerging technique is expected to bring a global change in the ratio of male to female progeny resulting in quick enhancement in global milk

production. With this technique, it is expected that much of the small and marginal farmers would be benefited immediately avoiding their spending on unproductive males. In recent past, some illuminating findings indicated an obligatory role of female reproductive tract in selecting spermatozoa and diverted the long-standing attention on Y chromosome for skewing sex ratio. Earlier, female reproductive tract was exclusively recognized as a site for fertilization, but recent advances in non-mammalian species, traced the role of female in selecting a particular sex of spermatozoa through an unexplored phenomenon called cryptic female choice (Fitzpatrick and Lüpold 2014; Holt and Fazeli 2015). In this line, oral feeding of some exogenous biochemicals has widened the approaches of skewing sex ratio towards male or female. These includes diets enriched with low fat and high carbohydrate (Rosenfeld and Roberts 2004), high omega 6 FA (Fountain et al. 2008; Green et al. 2008; Gulliver et al. 2013), calcium and magnesium (Schmidt and Hood 2012; Arangasamy et al. 2015). On the other hand, Aulakh (2008) also reported that the administration of sodium ethanoate before insemination in cattle skewed the sex ratio towards female. Although the evidence of oral feeding of biochemicals has shown to skew sex ratio, the clear mechanism behind skewing is poorly understood.

In this chapter, the methods used for enrichment of either X-bearing or Y-bearing sperm population, their merits and demerits, and the concept of female influencing the sex of the offspring are discussed.

14.2 Evolution of Sperm Selection Methods

Nitte stevens and Edmund Beecher discovered the difference in the chromosome composition of male and female in insects (Brush 1978). They demonstrated that female possesses XX while male possesses XY chromosomes. Further investigation revealed that spermatozoa produced by males contain either X or Y chromosomes and not both (Stevens 1905; Wilson 1905). Since then, various methods were employed to separate X and Y spermatozoa in order to pre-select the spermatozoa and to skew sex ratio towards a particular sex in many species of animals including human beings. The difference between X and Y chromosome bearing spermatozoa has been widely studied to achieve their separation; the volumetric differences between X and Y sperm (X sperm is larger than Y sperm; Cui and Matthews 1993), Y sperm moves faster than the X spermatozoa, the differences in the charge of spermatozoa, i.e. X spermatozoa are more negatively charged than the Y spermatozoa (Mohri et al. 1986), immunological differences (Hendriksen 1999), difference in DNA content; X spermatozoa contain 2.8–4% more DNA in comparison with Y chromosome bearing spermatozoa (Johnson 2000). Although different methods have been tried to separate X and Y spermatozoa, the most successful one is based on variation in DNA content between X and Y chromosome bearing spermatozoa. Methods and factors involved in skewing a particular sex are given below (Fig. 14.1).

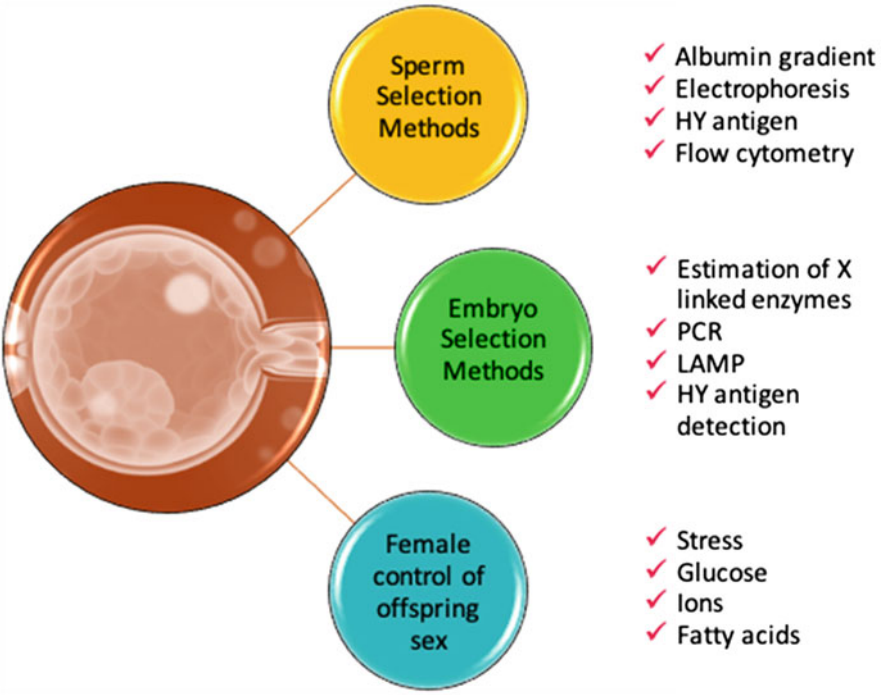

Fig. 14.1 Different methods and factors involved in offspring sex preselection

14.3 Sperm Selection Methods

14.3.1 Albumin Gradient

This technique is based on differential motility of X and Y chromosome bearing spermatozoa. This technique separated around 75–85% of Y sperm (Ericsson et al. 1973). The use of the albumin gradient selected spermatozoa in IVF has proved that it was successful in skewing sex ratio towards male in bovines. Few studies contradicted the report of skewing the sex ratio in bovines. However, this technique failed to skew the sex ratio in swine and horses. Although this technique has been able to skew the sex ratio towards males, the results were not consistent. Few studies postulated that serum albumin layering may not alter the proportion of Y-bearing sperm, but may alter the ability of Y-bearing sperm to fertilize the ovum. This technique was more successful in humans than other mammals (White et al. 1984).

14.3.2 Free Flow Electrophoresis

This technique is based on the differences in the surface antigenic charge of X and Y chromosome bearing spermatozoa. Net negative charge on X spermatozoa is relatively higher due to presence of neuraminic acid on the X sperm (Kaneko et al. 1984). Upon application of current, X sperm moves faster towards anode than the Y sperm due to its relatively higher negative charge (Mohri et al. 1986). This technique has been successfully applied in human (Kaneko et al. 1983; Engelmann et al. 1988; Ishijima et al. 1991), murine (Ishijima et al. 1991) and bovine (Masuda et al. 1989; Blottner et al. 1994). Blottner et al. (1994) verified different fractions using F body staining and double fluorescent in situ hybridization technique and enriched Y sperm in anodic fractions while X sperm in cathodic fractions. However, sperm sorted by this method loses motility hence could not be used for downstream applications (Gledhill 1988).

14.3.3 Identification of H-Y Antigen

H-Y antigen is a male specific glycoprotein secreted by Sertoli cells and expressed in spermatids and spermatozoa (Bradley 1989). Identification of H-Y antigen by immunoperoxidase staining revealed 50% of strongly stained spermatozoa, whereas 50% of relatively weaker spermatozoa. Such differences were extensively studied to distinguish the population of spermatozoa (Hendriksen 1999; Bennett and Boyse 1973). Subsequently, immunofluorescence staining also revealed the presence of HY antigen in postacrosomal region of head and weaker staining over flagellum in 50% of the observed spermatozoa (Bradley et al. 1987). Moreover, insemination with HY immunoaffinity column based skewed Y spermatozoa recorded 90% of male offspring (Bryant 1980). On the other hand, fluorescent assisted cell-sorted HY + ve and -ve spermatozoa using Feulgen stain revealed 80% of HY + ve spermatozoa had Y chromosome (Ali 1986). However, in contrast to these findings, Ohno and Wachtel (1978) demonstrated the presence of HY antigen on the membrane of both the population of spermatozoa. Thus, Prasad et al. (2010) concluded that HY antigen might not be a suitable marker for separation of X and Y spermatozoa.

14.3.4 Flow Cytometry Based Sorting of X and Y Spermatozoa

The DNA content of X chromosome is 2.8–4% more than the Y chromosome, which is the basis of this technique of separation of X and Y chromosome bearing spermatozoa with an accuracy of 90–92%. The stain bisbenzimide H 33342 trihydrochloride (Hoechst 33342) is used to stain sperm nuclei, which penetrates intact cell and binds to sperm DNA by van der Waals forces to AATT sequence in the minor groove of DNA (Teng et al. 1988). Upon excitation with UV laser (355 nm), Hoechst 33342 emits blue light leading to accurate measurement of

differences in the DNA content between X and Y spermatozoa (Seidel Jr and Garner 2002). Further, separation of dead spermatozoa from the viable ones was achieved by staining the spermatozoa with food colouring agent like FD&C Red No. 40 (Johnson and Welch 1999) or Yellow No. 6 (Sharpe and Evans 2009) which enters only in the spermatozoa with damaged membrane. The shape of the head in mammalian spermatozoa makes it readily oriented in sperm sorter using hydrodynamics in comparison with spermatozoa with angular or round head (Garner 2006).

Insemination of sex-sorted semen in rabbits yielded sex ratio of 94% of females and 81% male (Johnson and Pinkel 1986). Following this technique, many reproductive biotechnological tools such as intracytoplasmic sperm injection (Maxwell et al. 2004), in vitro fertilization (Matoba et al. 2014) and artificial insemination (Cran et al. 1995; Xu et al. 2009) were used to produce the sex pre-selected calf using bull sperm.

Many technical advancements have been done to increase the purity and viability of the separated spermatozoa. Three aspects were focused, viz. hardware, optics and electronics (Garner et al. 2013). The problems in the sperm orientation were overcame by development of novel nozzle tip that orients around 70% of the sperm head towards laser in contrast to 30% in conventional nozzles (Johnson and Welch 1999). Advancement in the nozzle apart from increasing the orientation also increased the sorting rate from about 600 sperm/sec in conventional sorter to 15,000 sperm/s (Rens et al. 1998). The exposure time of laser to spermatozoa needs to be minimized to reduce the damage caused due to the UV laser and was achieved by designing beam shaped lenses which further increase the efficiency of sperm sorting (Garner et al. 2013). Improvement in the detector circuits was essential to reduce the noise signal between X and Y peaks, this was achieved by making the response of detector non-linear and increasing the distance between X and Y peaks (Evans 2010). The mechanical pressure exerted during sorting at a speed of 55–60 mph was one of the important causes for damage of spermatozoa (Garner and Suh 2002), this was partially rectified by reducing the sheath pressure from 50 to 40 psi (Suh et al. 2005). The laser which was conventionally used, i.e. water-cooled continuous wave laser was replaced by pulsed lasers being air cooled reducing the need for expensive water coolers, thereby reducing the cost to 1/tenth of the original cost (Evans 2010). The problem in the purity of sorted spermatozoa was mainly due to the fact that coinciding spermatozoa was not able to differentiate properly and was getting wasted, the newer version of MoFlo SX XDP has overcome this problem by using digital technology, and the processing electronics in this machine can differentiate two closely placed spermatozoa, thus improving the yield of sorting (Sharpe and Evans 2009).

Morphokinetics of sex-sorted spermatozoa revealed highly variable motility pattern in vitro, the motility was significantly lower in sex-sorted semen in comparison to its counter parts within the same bull (Steele et al. 2020). The morphological changes have been observed in sex-sorted semen, the height, elongation, membrane roughness were higher, while form factor and circularity were lesser in comparison with non-sorted spermatozoa (Carvalho et al. 2018). The conception rates following

insemination with sex-sorted semen are 20–25% lesser than that of the conventional semen (Schenk et al. 2009; DeJarnette et al. 2010; Seidel Jr 2012, 2014). Pregnancy rate was 20% lesser in comparison to conventional spermatozoa (Hutchinson et al. 2013). Even increase in the dose of sexed semen from 2 to 4 million didn't significantly increased the conception rate or pregnancy rate (Lenz et al. 2017). Use of sex-sorted semen in in vivo embryo production revealed the reduction in number of recoverable embryos in comparison to conventional semen (Mikkola et al. 2015). Reduced cleavage rates with stained-sorted (53.1%) and stained-unsorted sperm (59.9%) to that of unstained-unsorted control sperm (69.9%). Similarly, the blastocyst rate in in vitro fertilized oocytes with sex-sorted semen was 39% against 65% in conventional semen (Steele et al. 2020). Further, studies of the sex-sorted semen revealed alterations in structural and functional attributes of spermatozoa. These changes include compromised kinematics (Steele et al. 2020), membrane integrity (Spinaci et al. 2013), higher percentage of acrosome reacted spermatozoa (Carvalho et al. 2010), higher DFI (Gosalvez et al. 2011) and compromised ability to form sperm reservoir in the oviduct (de Oliveira Carvalho et al. 2018). High cost of technology, equipment, production, requirement of skilled and trained manpower, slow rate of sorting, more wastage of sperm and low pregnancy rates are few important demerits of this technique (Seidel Jr and Schenk 2008).

14.4 Embryo Selection Methods

14.4.1 Estimation of X Linked Enzymes

X chromosome linked enzymes have been successfully used to identify the sex of the embryo before transferring into the recipient female. The activity of enzymes hypoxanthine phosphoribosyl transferase (HPRT) and glucose-6-phosphate dehydrogenase is higher in female embryos in comparison to male embryos. Few studies have used these enzymes for determination of embryo sex and got variable results. This variation in the enzyme activation was limited to the time of embryo genome activation which occurs between 8 and 16 cell stage, but if the assay is performed at the time of blastocyst when the X chromosome is inactivated, it results in bimodal results, while performing the assay at 8 cell stage or prior will give more accurate results.

14.4.2 Polymerase Chain Reaction

The determination of Y linked genes in the embryo has been used to sex the embryo prior to transfer with good efficacy, this process includes isolation of blastomeres from the embryo, amplification of the DNA from these blastomeres using sex-specific probes and interpretation by using electrophoresis. Sry or amelogenin (AMEL) gene is used for the determination of the embryo sex; amelogenin is a gene

located on both X and Y chromosome but amplifies the fragments of different sizes for AMELX and AMELY genes hence could be used to identify the sex of both X and Y sperm using a single set of DNA probes. The use of PCR for determination of embryo sex has been successfully carried out in cattle, sheep, goat, horse and pigs. The accuracy of predicting the sex is higher in PCR method in comparison to other methods. The false positives in this method were due to contamination of DNA during the procedure. The pregnancy rate in this method has been reported to be more than 40% in frozen thawed mouse embryos.

14.4.3 Loop-Mediated Isothermal Amplification (LAMP)

LAMP is a variation of the PCR where the amplification of the DNA from the blastomeres is amplified in isothermal condition using the enzyme DNA polymerase and four sets of primers along with the special set of primer known as loop primer which accelerates the lamp reaction. The inner and outer primers form a stem-loop DNA followed by amplification of large amount of DNA by auto-cycling reaction (Hirayama et al. 2013). The amplification is determined by the turbidity due to precipitation of amplified DNA by magnesium pyrophosphate, a precipitate of DNA synthesis using turbidity meter. This technique has been successfully used in bovines for determination of embryo sex, and a commercial kit has been developed using a specific DNA sequence repeat designated as S4. The LAMP-based sexing method has good sensitivity, accuracy, quick and easy to perform for cattle embryo sexing at field level (Hirayama et al. 2013).

14.4.4 Detection of HY Antigen

Histocompatibility antigen or HY antigen is a male specific surface antigen present on the somatic cells of heterogametic sex of all species. This can be used to determine the sex of embryo by either cytotoxicity assay or immunofluorescence assay. In cytotoxic assay, the polyclonal antiserum developed against HY antigen and in presence of complement the embryos are incubated with antiserum, the male embryos are destroyed while the female embryos develop normally. Immunofluorescence assay involves detection of HY antigen on the male embryo, which could be demonstrated from 8 cell embryo stage to the blastocyst stage. The accuracy of detection of male embryos in this technique is about 80–85% in different species of animals, indirect fluorescent technique enables transfer of fresh embryos without much compromise in the quality of embryo. The false positive in this technique is because the HY antigen is not absolutely sex specific leading to the cross reactivity.

14.5 Female Control of Offspring Sex

Even though the genetic sex of the spermatozoa has been determined by the sex of the spermatozoa that fertilize the oocyte, i.e. X chromosome bearing sperm or Y chromosome bearing sperm, the decision of which sperm to be allowed to fertilize the oocyte could be with the female. Females exert a cryptic mechanism which enables her to select the spermatozoa with desirable phenotypic attributes and incubate them in the isthmus (sperm reservoir) known as "cryptic female choice" (Holt and Fazeli 2010). A study has shown that oviduct differentially responds to the spermatozoa bearing X or Y chromosome bearing spermatozoa and further highlighted the importance of female in the selection of offspring sex (Almiñana et al. 2014). The fact that various factors including diet, environment and stress to the female skew the sex ratio towards a particular sex has been reported. Proposed mechanisms by which the female could control the sex of the offspring are indicated in Fig. 14.2.

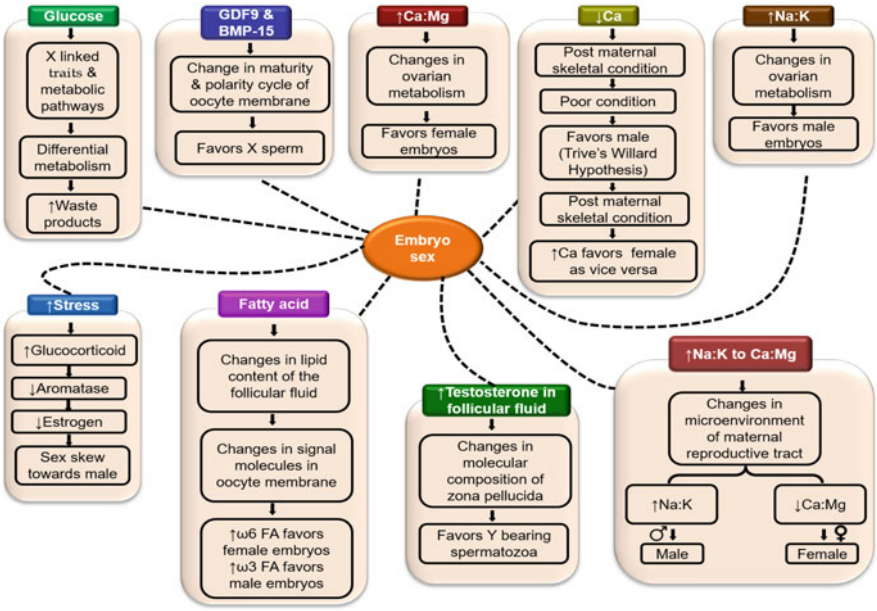

Fig. 14.2 Different mechanisms proposed for skewing sex ratio in different species (Schmidt and Hood 2012; Green et al. 2016; Behnam-Rassouli et al. 2010; Rosenfeld et al. 2003; Arangasamy et al. 2015)

14.6 Theories Supporting Female Control of Offspring Sex

14.6.1 Trivers–Willard Hypothesis

"The sex of the offspring is determined by the condition of female" was proposed by Trivers and Willard (1973). The experiment conducted on Caribou showed that female in good condition tends to produce more male offspring as they have more chances to father the offspring, whereas the females in poor condition tend to produce female. This theory insists the importance of parental investment on the sex of offspring. Different possible explanations have been provided to support this hypothesis. Douhard (2017), in support of Trivers–Willard hypothesis, opined that the females in good condition have more glucose. Female embryos, owing to increased metabolism, produce more toxic products through pentose phosphate pathway which is facilitated by X linked protein which may prove detrimental for survival of female embryos (Edwards et al. 2016). Poor conditioned females will be under stress and produce more glucocorticoid and higher free radicles; the male embryos are more susceptible to the oxidative damage; hence, the poor condition females have an environment that is detrimental to the male embryos hence skewing the ratio towards the female (Navara 2010).

14.6.2 Maternal Dominant Hypothesis

Grant (1996) proposed maternal dominance hypothesis; dominant mother produced more serum testosterone hence produce more sons than daughter. Contrasting results in primates indicated that the dominant or higher ranked mothers produced more females than males probably in these species daughters could repay the maternal investment better than the males (Meikle et al. 1984). Maternal testosterone specially in the follicular fluid might influence the molecular composition of zona pellucida resulting in reduced receptivity to Y bearing spermatozoa further skewing the embryo sex towards female (Grant and Chamley 2010).

14.7 Maternal Factors Affecting Sex Ratio

14.7.1 Stress

Since long time various stressors have been reported to skew sex ratio in species exhibiting environmental sex determination and genetic sex determination (Capel 2017). The stressors are mediated by glucocorticoids mostly cortisol (Romero 2004). It is also proven that subordinate females produce more glucocorticoids in comparison to dominant ones, hence supporting the maternal dominant hypothesis (Abbott et al. 2003). In howler monkeys, when the cortisol concentration was below the threshold of 200 ng/g at the time of conception consistently produced females while more than 200 ng/g produced males (Rangel-Negrín et al. 2018). Increased levels of

glucocorticoids in mammals bound to its receptors, further reduced the aromatase which is responsible for conversion of androgens to oestrogen. Reduced oestrogen and increased androgens favoured development of male embryo skewing the sex ratio towards male (Geffroy and Douhard 2019). All these findings support the maternal dominant hypothesis and also indicate that higher level of stress skews the sex ratio towards male.

14.7.2 Maternal Diet

Maternal diet during peri-conceptual period in different mammals has been shown to skew the sex ratio towards a particular sex. A recent literature entitled "you are what your mother eats" emphasized the role of maternal diet on the offspring based on the retrospective studies, which revealed consumption of more cereals led to male child (Mathews et al. 2008). Different dietary modifications and its influence on sex ratio are summarized in Table 14.1. Among different diets which are said to skew the sex ratio, carbohydrates (Cameron 2004), fatty acids (Green et al. 2008) and ionic diets

Table 14.1 Effect of diet on skewing sex ratio of offspring towards a particular sex

Nutrient	Species	Offspring sex skewed towards	Reference
High-fat diet	Mice	Male	Rosenfeld et al. (2003)
Low-fat, high carbohydrate	Mice	Female	Rosenfeld and Roberts (2004)
Omega 3 FA	Mice	None	Fountain et al. (2008)
Omega 6 FA	Mice	Female	Fountain et al. (2008)
PUFA	Sheep	Male	Green et al. 2008
Omega 6 FA	Sheep	Female	Gulliver et al. (2013); Clayton et al. (2016)
Glucose	Field voles	Male	Helle et al. (2008)
Dexamethasone	Mice	Female	Cameron et al. (2008)
Calcium and magnesium	Rat	None	Vahidi and Sheikhha (2007)
Low calcium diet	White-footed mice	Female	Schmidt and Hood (2012)
Calcium and magnesium	Rat	Female	Arangasamy et al. (2015)
Calcium and magnesium	Rats	Female	Oun et al. (2016)
Sodium and potassium	Rats	Male	Vahidi and Sheikhha (2007)
Chicory-increased sodium and potassium	Rats	Male	Behnam-Rassouli et al. (2010)
Sodium and potassium	Rats	Male	Oun et al. (2016)
Omega-6 PUFA	Cattle	Male	Marei et al. 2018
Sodium, potassium and phosphorus	Ovine	Male	Alhimaidi et al. 2021
Calcium and magnesium	Ovine	Female	Alhimaidi et al. 2021

(Alhimaidi et al. 2021) are extensively studied. However, the mechanisms by which these factors skew the sex ratio towards particular sex are not fully understood.

14.7.2.1 Dietary Ions

Minerals play a vital role in maintaining homeostasis in the animal, various minerals, viz. calcium, magnesium, sodium and potassium have said to have multiple roles. The effect of these minerals has been extended to skewing the sex ratio in many species including cattle (Stolkowski and Lorrain 1980), sheep (Alhimaidi et al. 2021), sow (Bolet et al. 1982), rat (Arangasamy et al. 2015) and mice (Schmidt and Hood 2012). The ionic concentration of these minerals modulates the pH in female reproductive tract, specifically at the fallopian tube, which is considered to be the store house of spermatozoa and also the site of fertilization (Arangasamy et al. 2015). The changes in the pH of female reproductive tract specifically in follicular fluid towards basic environment have said to attract more of X chromosome bearing spermatozoa which is negatively charged (Grant and Chamley 2010). The concentration of these ions in the oviduct has said to modulate the secretion in the exocrine glands of the fallopian tube. This modulation may further influence the spermatozoa of a particular sex in the isthmic reservoir to compete to bind to the oocyte. Additionally, the change in the constitution of the fallopian tube might influence the oocyte to change its receptor on the zona pellucida to bind to either X or Y spermatozoa (Alfageeh and Alhimaidi 2013).

Arangasamy et al. (2015) fed the Wistar rats with 2% calcium along with 0.4% magnesium from 15 days prior to mating to 7 days postmating and reported a significant skewing of sex ratio towards female. They opined that these ions modulated the expression of BMP-15 and GDF-9 in the oocyte, which might have affected the oocyte maturation and polarity cycle of the oocyte membrane to attract the spermatozoa of a particular sex. Further, changes in ionic pH of the fallopian tube could have induced the membrane changes in the gametes leading to skewing the sex ratio. Schmidt and Hood (2012) fed low-(0.1%) and standard calcium diets (0.85%) to mice for about 75 weeks and observed the reproductive output. The mice fed with low calcium diet skewed the sex ratio towards female while the mice fed with standard diet no sex ratio was skewed. They have observed that mice with low calcium had poor maternal skeletal condition and could be compared with the mothers with low condition producing female-biased litters (Trivers and Willard 1973).

Oun et al. (2016) observed that feeding of minerals has skewed the sex ratio. They studied the effect of calcium and magnesium diet and sodium and potassium diet on sex ratio in rats. They observed 60% females in calcium and magnesium group while only 4.19% females in sodium and potassium group. Vahidi and Sheikhha (2007) compared the sodium and potassium with calcium and magnesium diet in skewing sex ratios, they observed that sodium and potassium diet skews the sex ratios towards male while calcium and magnesium diets skewed the sex ratio towards female offspring. They concluded that changes in the ovarian metabolism due to changes in the ratio of calcium: magnesium and sodium: potassium might influence the sex of the offspring. Behnam-Rassouli et al. (2010) observed the effect of chicory

extract on the sex ratio of rats. They observed increase in serum sodium and potassium ions. The ratio of monovalent cations and divalent cations has been significantly higher in chicory fed group in comparison to the control. They claimed that chicory skewed the sex towards male litter by acting on monovalent cation, i.e. sodium and potassium.

Alhimaidi et al. (2021) fed sheep for 30 days prior to mating with diets rich in sodium, potassium and phosphorous to one group and diets rich in calcium and magnesium to another group. The results revealed the diets rich in sodium and potassium yielded 77% of male offspring while the calcium and magnesium rich diets resulted in 73% of female offspring. They hypothesized that the results might be due to the modulation of micro-environment of maternal reproductive tract by differential availability of calcium, magnesium, sodium and potassium in the treatment groups.

14.7.2.2 Fatty Acid

Free fatty acids or polyunsaturated fatty acids have proved to alter the sex ratio in many species including cattle, sheep (Green et al. 2008) and mice (Rosenfeld et al. 2003). Among the fats, omega 6 fatty acid skewed the sex ratio towards female (Rosenfeld et al. 2003) while omega 3 fatty acids favoured male offspring (Green et al. 2008). Marei et al. (2018) proposed that the PUFAs directly act on the female reproductive tract more specifically on the follicle to alter the sex ratio. Linoleic acid constituted about 1/third of follicular fluid fatty acid component (Homa and Brown 1992). The content of fatty acid in the lipid of oocyte and cumulus cells varies upon dietary manipulation (Zachut et al. 2010). The changes in the fatty acid composition and its incorporation into the cellular structure modify the signalling molecules as well as properties of the cell membrane (Wathes et al. 2007). A recent study involving AnnexinA1 (anti-inflammatory protein) knockout mice resulted in increased number of female pups in litter even though the sex ratio in male sperm didn't vary (Hebeda et al. 2018). Annexin A1 is involved in inhibition of phospholipase A2 activity, which is essential in the process of biosynthesis of prostaglandins (Liu et al. 2007). PUFAs have shown to affect the expression patterns of cell associated Annexin A2 in human umbilical veins, however, the effect of annexin A2 on sex ratio has not been studied (Park et al. 2014).

14.7.2.3 Glucose

Glucose is one of the important factors said to influence sex ratio in mammals (Cameron 2004). Glucose acts peri-conceptually to alter the sex ratio (Edwards et al. 2016). Cameron (2004) proposed that the pre-implantation levels of glucose affect the sex ratio of the resultant offspring. The interaction with the X linked traits proteins and the metabolic pathways (Gutierrez-Adan et al. 2001) resulted in higher metabolism rate of female embryos via pentose phosphate pathway results in increased production of reactive oxygen species (ROS) which was detrimental for the survival of the embryo. In contrast, the lesser X linked proteins in male embryo resulted in production of optimum quantity of reactive oxygen species which supports its growth and development (Green et al. 2016).

14.8 Perspective and Prospective

Producing young ones of desired sex has always been the desire of livestock farmers. Several attempts have been made to develop a method that efficiently separates bovine semen into fractions containing higher concentrations of X- or Y-bearing sperm. Among these methods, use of sexed semen for artificial insemination is recognized as more pragmatic and easy way to pre-select the sex of the offspring. Selective use of sexed semen in breeding will increase the genetic progress from the daughter-dam path and help in producing good male germ plasm from elite bulls for future breeding. However, sorting pressure, speed, electrical deviation, laser radiation all lead to membrane alteration and pre-capacitation like changes in the sorted sperm leading to reduced fertility. Future challenges with this technique could be production of sorted semen at economic cost with high rate of sorting and good pregnancy outcome.

Identification of sex-specific surface markers would allow for immunological separation or targeted immobilization of spermatozoa of one sex and thus enable a safer, more widely applicable and higher throughput means of sperm sorting. Targeted killing, i.e. killing of unwanted sex bearing spermatozoa either at the production site itself or after ejaculation could also be an option. Since it is shown that the oviduct is capable of discriminating X- and Y-bearing spermatozoa, and the micro-environment of the oviduct facilitates binding of a particular sex spermatozoa, this approach offers scope to modulate the oviduct milieu at the time of insemination to facilitate establishment of sperm reservoir with a particular sex chromosome bearing spermatozoa. However, clear cut cause and effect experimental proofs are required before using this approach to skew the sex ratio. In long run, developing designer bulls that produce only one type (either X or Y) of spermatozoa by knocking out the other type is also possible in future.

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Genomic Selection for Fertility in Bovines

15

Jayakumar Sivalingam, M. R. Vineeth, Anshuman Kumar, Kamaraj Elango, and Indrajit Ganguly

Abstract

The fertility of dairy bovines affects the genetic improvement and economical sustainability of dairy herds. In the past, genetic evaluation of bovine traits was exclusively based on the information from phenotype and pedigree. Traditional selection schemes selected predominantly for milk yield often at the expense of other traits, including fertility. With the advent of genome wide DNA markers, low-cost genotyping technology, and development of suitable statistical methods, genomic selection came into picture. The most profound changes due to genomic selection in dairy cattle have been demonstrated for fertility traits. The use of genomic selection has almost doubled the rate of genetic gain in dairy cattle in several countries. These changes are achieved mainly due to the acquired capability to predict an animal's performance at an earlier age along with improved accuracy, which in turn results in the reduction of the generation interval and increase in selection intensity.

Keywords

Fertility · Daughter pregnancy rate · Sire conception rate · GBLUP

J. Sivalingam (✉)
ICAR-Directorate of Poultry Research, Hyderabad, Telangana, India

M. R. Vineeth · A. Kumar
Department of Animal Genetics and Breeding, Faculty of Veterinary and Animal Sciences, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

K. Elango
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

I. Ganguly
ICAR-National Bureau of Animal Genetic Resources, Karnal, Haryana, India

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15.1 Introduction

Fertility is a measure of reproductive success and is one of the most important traits in animal production. Darwash et al. (1997a, b) defined dairy cow fertility as "the ability of the animal to conceive and maintain pregnancy if served at the appropriate time in relation to ovulation." The fertility in males and females is measured by various traits. The fertility of dairy bovines influences the genetic improvement of dairy herds and economical sustainability of the dairy industry. In the old days, genetic evaluation of bovine dairy traits was exclusively built on the phenotype data recorded and pedigree information. Traditional breeding schemes selected primarily for milk production traits frequently ignoring other traits, including male and female fertility. With the advent of genome wide DNA markers, low-cost genotyping technology and development of suitable statistical methods, genomic selection (GS) came into picture. Many countries like Great Britain, United States, Canada, Australia, New Zealand, France, Germany, Netherlands, Belgium, Ireland, Finland, Sweden, and Denmark have successfully executed GS programs. Most of the genomic evaluation schemes focus mainly on female fertility, and there are reports about the successful genomic prediction for male fertility. Apart from cattle, there is limited studies in other bovines regarding genomic evaluation for reproductive traits. The coming years will see more extensive use of GS for fertility in cattle and buffaloes with more emphasis on novel fertility phenotypes which can explain the biological process of reproduction in a better way as well giving due weightage to the male fertility traits.

15.2 Marker-Assisted Selection and GS

In the old days, dairy bovine genetic evaluation was exclusively based on the data from phenotypic measurements and animal pedigree. But after the introduction of DNA markers and genotyping technology, marker-assisted selection (MAS) (Dekkers and Hospital 2002) came into picture. MAS indirectly selects individuals by applying the information of markers which are associated with quantitative trait loci (QTL). The MAS can be explained as a process consisting of two major steps: (1) detection and mapping of genes/QTLs responsible for the traits under study, (2) inclusion of the QTL data within the Best Linear Unbiased Prediction (BLUP) for the estimation of breeding values (Fernando and Grossman 1989). Still, practical utilization of MAS in bovine breeding schemes has not been successful. It was mainly due to reduced number of markers available, and the economic traits were regulated by a vast number of genes or QTL with minimal consequences.

During the early days of twenty-first century, Meuwissen et al. (2001) put forwarded a new approach known as the genomic selection (GS). It is a modified version of MAS, where predictions are made using data from a large number of genome wide markers. The basic assumption of this method was that the genome wide markers were in linkage disequilibrium (LD) with QTL, and hence a greater fraction of the additive genetic variance will be elucidated by these genome wide

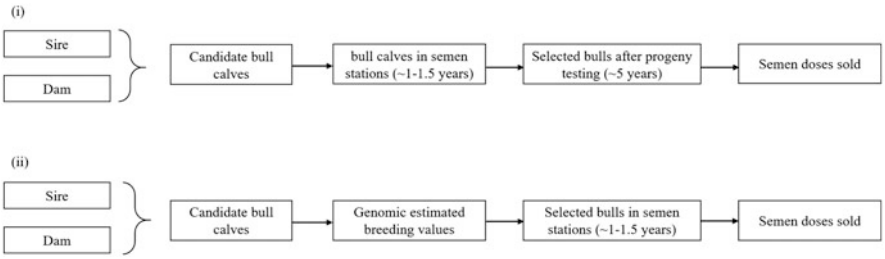

Fig. 15.1 Comparison of (**i**) conventional progeny testing with (**ii**) GS scheme

markers. Many simulation studies found high accuracy of prediction using this approach, and Schaeffer (2006) established that such higher accuracies can possibly twofold the present genetic gains obtained by dairy cattle progeny testing schemes. As a result, GS was progressively embraced as the method for genetic evaluation in dairy cattle breeding schemes by developed countries and subsequently by developing countries also. The implementation of GS includes the subsequent points:

1. Creation of a reference population (also known as training population), where animals are genotyped for genome wide markers and the phenotypic information for traits are available;
2. Development of suitable statistical method for estimating genomic estimated breeding value (GEBV).
3. Genotyping the candidate animals for genome wide markers.
4. Finding genomic estimated breeding value (GEBV) of candidate animals using the appropriate statistical method.
5. Selection of candidate animals based on GEBV.

The most important aspects of the GS are its ability to reduce the generation interval significantly. Figure 15.1 demonstrates the difference of conventional bull evaluation with genomic evaluation.

With respect QTL studies in cattle, there has not been any significant validated QTLs which can be used in MAS for production and fertility traits except DGAT1 gene which affects fat content in milk (Grisart et al. 2001). A comprehensive study of QTL, markers, and candidate genes for male and female fertility traits can be found in Cochran et al. (2013a, b), Fortes et al. (2013), Ortega et al. (2017), Buzanskas et al. (2017), Taylor et al. (2018), and Sweett et al. (2020).

15.3 Measuring Fertility in Bovines

The dairy bovine fertility comprised of two components, the female and male fertility.

15.4 Female Fertility Traits

The fertility in cows is measured in variety of ways/traits. The most commonly used ones are described here.

15.4.1 Age at Puberty

Age at puberty is defined as the age at which the heifers first ovulate. This can be detected by estrus/heat signs, hormonal assays, or by ovarian ultrasonography. The age at puberty is one of the important fertility traits.

15.4.2 Age at First Calving

It is defined as the age at which the animal calves for the first time. The age of the animal at first calving is very important with respect to lifetime production.

15.4.3 Cow Conception Rate (CCR)

The United States Department of Agriculture (USDA) has defined CCR as the proportion of inseminated cows that become pregnant at each service. It measures the ability of lactating cows to conceive. PTA CCR of 1.0 leads to a 1% increase in cow conception rate compared to a CCR of 0.

15.4.4 Heifer Conception Rate (HCR)

It is similar to CCR. It measures the ability of heifers to conceive. It is defined as the percentage of heifers inseminated that subsequently become pregnant at every single service.

15.4.5 Daughter Pregnancy Rate (DPR)

The DPR is defined as the proportion of non-pregnant cows that conceive during each 21-day period after the voluntary waiting period. Predicted transmitting ability (PTA) estimates the amount of superior genetic merit of a bull which will be passed onto its offspring. A PTA DPR of "1" for a particular sire suggests that daughters obtained from that sire have 1% more chance to conceive during a particular estrus cycle than a sire with a DPR of zero. Every rise of 1% in PTA DPR is equivalent to a reduction of about 4 days in PTA days open (Norman et al. 2009). DPR measures the cow's ability to begin cycling, show estrus, conceive and maintain pregnancy and is

highly correlated with productive life. The heritability of DPR is low (Averill et al. 2004; Pryce et al. 2004).

15.4.6 Daughter Calving Ease (DCE)

DCE can be defined as the ability of daughters of a particular bull to give birth to calves that are calved more easily than calves produced by an average cow. It measures the ability of a cow to calve easily. Lower daughter calving ease is highly correlated with a long productive life.

15.4.7 Days Open (DO)

It defined as the period (measured as average number of days) from calving to successful conception. In case of cows which are failing to conceive, it is measured as the period from calving to culling. If the days open is too prolonged, the calving interval will get prolonged, less no. of calving will be obtained in cow's lifetime and ultimately less lifetime production.

15.4.8 Calving Interval (CI)

It is defined as time period (measured in days or months) between the birth of a calf and the birth of a successive calf, both calved by the same cow.

15.5 Bull Fertility Traits

Male fertility is constituted of the sperm's fertilizing ability along with the embryo viability (Azzam et al. 1988). A significant percentage of reproductive failure in dairy cattle can be attributed to sub-fertility in service sires (DeJarnette et al. 2004). A service sires whose semen does not conceive his mate leads to economic losses by lengthening calving intervals, increasing cost of replacement and resulting in additional insemination charges (Hyppanen and Juga 1998).

Bull fertility can be evaluated on physical (scrotal circumference), physiological (semen quality parameters), or success rate (NRR, conception rate) basis (Miglior et al. 2005).

15.5.1 Scrotal Circumference (SC)

SC is physical trait which is frequently used as a good indicator of the semen production potential of the bull. It is measured around broadest part of the testicles

using a circular tape (Whittier and Bailey 2009). The scrotal circumference at maturity varies among breeds and different bovine species.

15.5.2 Semen Production and Quality Measures

Semen Volume is the total volume of the single ejaculate from a male. It is often expressed in milliliters.

Semen Concentration is the total number of spermatozoa found in the ejaculate. It is measured using a colorimeter. The semen concentration is often denoted as millions per milliliter (Suchocki and Szyda 2015).

Sperm Motility is used to describe the capability of the spermatozoa to travel progressively forward. Sperm motility is often known as progressive motility when it is being evaluated using a computer-assisted semen analyzer. This term is frequently used to describe whether the spermatozoa in the ejaculate are swimming in straight lines or in circles. The sperm motility is denoted as a percentage value. The ejaculate sample is observed under a microscope and 100 spermatozoa are counted and classified as moving (motile) or non-motile (Senger 2012; Butler et al. 2020).

Mass activity is a subjective score given for the ejaculate based on the scale followed by the collecting station. Mass activity is also known a mass motility. It is observed by placing a drop of the ejaculate in a warm glass slide and observed under low power microscope without a coverslip. The collective movement of the spermatozoa is evaluated and scored. Normally, it is scored based from 0 to 4 or 0 to 5. Always a higher mass activity is preferred. (Ducrocq and Humblot 1995).

Percentage of normal spermatozoa is the measure of normal spermatozoa. It is the number of spermatozoa with an acceptable or normal morphology among the hundred spermatozoa evaluated.

Sperm Abnormalities is the measure of abnormal spermatozoa or spermatozoa with undesirable characters in the ejaculate. It is denoted as percent abnormalities. The most common abnormalities seen are spermatozoa with an abnormal head, tail, and cytoplasmic droplet (Butler et al. 2020).

Most of the trait has low to moderate heritability while scrotal circumference has moderate to high heritability.

DeJarnette et al. (2004) explain the various measure of fertility based on success rate as follows:

15.5.3 Non-return Rate (NRR)

It is defined as the percentage of cows that are not successively re-bred within a definite period of time after a particular insemination. The specific time period is usually between 60 and 90 days in USA (Nadarajah et al. 1988; Miglior et al. 2005), 56 days in Europe and Canada (Amann and DeJarnette 2012). In case of NRR, there is chance of errors introduced by improper identification of cows in heat. Moreover, errors also creep in if the repeat services are not recorded properly. The absence of

cross-checking systems to ensure if the animal is still present in the herd or not is essential for efficient NRR calculation. Hence, non-return calculations are prone to high error rates. The NRR are measured for each AI sire.

15.5.4 Conception Rate (CR)

CR is the same as the fertilization rate. It is defined as the percentage of females identified as pregnant in a particular time interval of 25 or 60 days after AI. Here, the pregnancy diagnosis is done by the methods such as per-rectal palpation, ultrasonography, and hormonal assay. The disadvantage with CR is that it fails to account for subsequent loss of pregnancy due to embryo or fetal death. Yet, for the determination of sire fertility potential, CR is associated with the least amount of error.

15.5.5 Estimated Relative Conception Rate (ERCR)

The United States' Dairy Records Management System introduced ERCR as trait for estimation of fertility of service sires in 1986 (Clay and McDaniel 2001). The basis of this phenotypic trait evaluations was NRR to first service. The NRR for the first service was calculated on a 70 day non return basis. The ERCR provided to dairy farmers for a particular AI sire with sufficient inseminations data so as to generate an accurate prediction of impending successful breeding.

15.5.6 Sire Conception Rate (SCR)

ERCR was replaced by SCR which is a predictor of bull fertility expressed as a relative conception rate. It is reported as a percentage value. This phenotypic bull fertility trait was the outcome of research works by Kuhn et al. (2006, 2008) and Kuhn and Hutchison (2008). SCR is calculated as the differential probability of a particular bull's semen dose leading to a pregnancy compared with the average of all other bulls' semen doses which could have been used for insemination. Only the artificial insemination data from first seven breeding with recorded outcomes as a success or failure are included in SCR calculation. An average bull has an SCR of 0.0%. A bull with an SCR of 3.0% is expected to have a 3% higher conception rate than an average bull and a 6% higher conception rate than a bull with an SCR of −3.0%. The

started national evaluation for service-sire conception rate in August 2008 and is carried out by the Animal Improvement Programs Laboratory (Norman et al. 2008).

15.6 Statistical Methods for Genomic Prediction

Meuwissen et al. (2001) proposed GS to predict more accurate breeding values by expanding the design of integration genome wide marker information into BLUP (Fernando and Grossman 1989; Lande and Thompson 1990; Haley and Vischer 1998). For this purpose, Meuwissen et al. (2001) used Bayesian models which provide SNP effects and direct genomic values (DGVs) based on the combined analyses of genotypes and phenotypes. This method was easy to be modified to use pseudo-phenotypes like estimated breeding values or progeny deviations when the genotyped animals are bulls (Lourenco et al. 2020).

Similarly, an alternate method called the genomic BLUP (GBLUP) which can generate the predictions for genotyped animals on the basis of genomic relationships instead of pedigree relationships was proposed (VanRaden 2008).

The GBLUP or Bayesian methods had a drawback that even after their use, another extra processing step was required. This was necessary to account for the pedigree information. So, the standard BLUP evaluation continued to be in use. As many steps are required to generate the genomic estimated breeding value (GEBV), this set of methods were called multistep methods. The predominant benefit of this multistep method is that the cost is lower and the traditional BLUP evaluation is kept unchanged. Despite all these, the multistep method has some drawbacks like DGVs are only produced for simple models (like single trait models, non-maternal models), and it requires pseudo-phenotypes that are difficult to obtain and need approximations (Legarra et al. 2014).

In 2009, a new method was proposed by Misztal et al. (2009) which integrates pedigree information, phenotypic and genotypic data into a single evaluation scheme. This method is known as the single-step genomic BLUP (ssGBLUP), and it uses a realized relationship matrix (which is a combination of pedigree and genomic relationships) in place of the pedigree relationship matrix in the traditional BLUP. Here, the pedigree relationship can be taken as a priori relationship, whereas the genomic relationship can be taken as the observed relationship (Legarra et al. 2009). Imputing the genomic data to the non-genotyped animals can be carried out through deriving the combined distribution of genomic and pedigree relationships. Thus, in ssGBLUP, non-genotyped animal's pedigree relationships are enhanced by their relative's genomic data. After a decade of GS, ssGBLUP has emerged to be the most widely used statistical method for genomic prediction (Lourenco et al. 2020).

Statistical methods have gradually advanced to single-step, multi-trait, multi-breed, or other more complex models (Mantysaari et al. 2019). Multi-breed models provide very little benefit for pure breeds unless they are closely related, but crossbred selection may be benefitted. Most of the official prediction schemes use multiple regression on individual SNP, which works well if numbers of SNP or linkage disequilibrium are high (Calus et al. 2008). Meta analysis methods at international levels like genomic multi-trait across-country evaluation (MACE) were developed to merge and convert genomic predictions from each country onto the scales of all other countries (Sullivan 2019).

Reliability of predictions is having been moderate with large genotyped populations. Prediction reliabilities averaged 71% for Holsteins and 65% for Jerseys (VanRaden and O'Connell 2018). There is no consensus on a uniformly best method for GS (Weller et al. 2017).

15.7 GS for Female Fertility Traits

GS programs include evaluation of female fertility traits. The Interbull's genomic MACE provides evaluation for female fertility in Brown Swiss, Guernsey, Holstein, Jersey, Ayrshire, Nordic Red and Simmental cattle breeds. A summary of individual GS studies is presented in Table 15.1.

15.8 GS for Male Fertility Traits

Although the fertility depends on both male and female, most of the genomic evaluation schemes focus mainly on female fertility. There are reports of successful genomic prediction for male fertility like SCR (Abdollahi-Arpanahi et al. 2017; Nani et al. 2019; Rezende et al. 2019; Mantysaari et al. 2019) and scrotal circumference (Boddhireddy et al. 2014; Neves et al. 2014; Lopes et al. 2018; Piccoli et al. 2020). A brief summary of the GS studies for male fertility traits has been given in Table 15.2.

15.9 Outcomes/Results of GS for Fertility Studies

The use of GS has increased the rate of genetic gain in dairy cattle in several countries to twice the existing gains. These changes are achieved mainly due to the acquired capability to predict an animal's performance at an earlier age along with improved accuracy, which in turn results in the reduction of the generation interval and increase in selection intensity (Garcia-Ruiz et al. 2016). Even though GS has been reported to reduce inbreeding, there are reports of increased inbreeding in GS programs (Forutan et al. 2018a, b; Doublet et al. 2019).

Garcia-Ruiz et al. (2016) have reviewed the results from nearly two generations of GS in the US Holstein cattle. The results showed an increase in yearly rates of genetic gain of about 50–100% for the yield traits and while it was three to four-fold for other traits like female fertility, herd life, and somatic cell score which are having low heritability. The period after the implementation of GS witnessed significant reductions in generation interval, particularly in the sire to bull (SB) and sire to cow (SC) paths. The SB generation interval decreased from around 7 years to less than two and half years, and the dam to bull (DB) generation interval went down from around 4 years to nearly two and half years. The most notable changes where found in low heritable traits, where genetic trends for fertility and other such traits changed from almost zero to large and favorable, ensuing in faster genetic improvement in fertility.

Table 15.1 A list of GS studies in female fertility traits of cattle

Trait	Breed	Models	Accuracy of prediction	References
Puberty (age at first corpus luteum)	Brahman and tropical composite	GBLUP	0.52 0.49	Engle et al. (2019)
	Composite	BayesA BayesB BayesC	0.237	Toghiani et al. (2017)
	Brahman and tropical composite	GBLUP	0.33 0.15	Zhang et al. (2014)
Corpus luteum score	Multi-breed population (Brahmans, droughtmasters, and Santa Gertrudis)	GBLUP BayesR	0.15–0.35	Hayes et al. (2019)
Age at first calving	Nellore	GBLUP BAYESCp IBLASSO	0.38 0.42 0.39	Costa et al. (2019)
	Nellore	BayesA BayesB BayesCp BLASSO BRR	0.24 0.23 0.33 0.24 0.38	Mota et al. (2018)
	Nellore	BayesC	0.64	Boddhireddy et al. (2014)
	Crossbred animals	Ss GBLUP ssGBLUPS1 ssGBLUPS2	0.30 0.30 0.26	Laodim et al. (2019)
	Gyr	GBLUP	0.38	Boison et al. (2017)
	Composite	BayesA BayesB BayesCp	0.15	Toghiani et al. (2017)
Calving to first service	Holstein	GBLUP	0.04	Tenghe et al. (2015)
Non-return rate	Holstein	ssGBLUP msGBLUP	0.39	Guarini et al. (2018)
Heifer pregnancy rate	Angus	BayesC	0.38	Saatchi et al. (2011)
	Nellore	BayesC	0.64	Boddhireddy et al. (2014)
Days open	Holstein	GBLUP	0.50	Forutan et al. (2018a, b)
Heifer pregnancy status	Composite	BayesA BayesB BayesC	0.64	Toghiani et al. (2017)
Calving ease Direct	Brangus	tsGBLUP ssGBLUP	0.45 0.34	Lopes et al. (2018)
	Angus	BayesC	0.62	Saatchi et al. (2011)

(continued)

Table 15.1 (continued)

Trait	Breed	Models	Accuracy of prediction	References
Calving ease Maternal	Brangus	tsGBLUP ssGBLUP	0.51 0.27	Lopes et al. (2018)
	Angus	BayesC	0.57	Saatchi et al. (2011)
Calving ease	Holstein	ssGBLUP msGBLUP	0.76	Guarini et al. (2018)
	Norwegian red	GBLUP BayesB MIXTURE	0.41 0.41 0.43	Luan et al. (2009)
Gestation length	Nellore	GBLUP BayesC BLASSO	0.71 0.72 0.72	Neves et al. (2014)
DPR	Holstein	BayesC	0.82	He et al. (2018)

Table 15.2 List of GS studies for male fertility traits

Trait	Breed	Models	Accuracy	Reference
SCR	Holstein (US)	RKHS	0.35	Abdollahi-Arpanahi et al. (2017)
	Holstein (US)	RKHS	0.34	Nani et al. (2019)
	Jersey (US)	RKHS	0.29	Rezende et al. (2019)
	Jersey (US) Jersey (Australia)	GBLUP	0.28 0.02	Rezende et al. (2019)
	Nordic red	GBLUP	0.26	Matilainen et al. (2018)
Scrotal circumference	Nellore	BayesC	0.59	Boddhireddy et al. (2014)
	Nellore	GBLUP BayesC BLASSO	0.68 0.72 0.72	Neves et al. (2014)
	Brangus	tsGBLUP ssGBLUP	0.71 0.63	Lopes et al. (2018)
	Braford and Hereford	tsGBLUP ssGBLUP	0.28 0.15	Piccoli et al. (2020)

The reports from France where the genomic evaluations of the dairy cattle breeds have been implemented since 2009 shows increased genetic gains and reduced generation intervals. The evaluations were for total genetic merit index -ISU (standing for Index Synthese Unique), which is an individual index which combines the production traits along with the functional and type traits, with appropriate weightage given depending on the breed breeding goals. GS resulted in a growth in the average yearly genetic gains of 71, 50, and 33 percent for, Normande, Montbeliarde, and Holstein bulls, respectively. While the generation intervals

found to be decreased by 1.9, 1.7, and 2 times, respectively. There was an increase in inbreeding rate for the Holstein breed (Doublet et al. 2019).

Scott et al. (2021) have reported that introduction of GS in Australian dairy herd has resulted in increase in g
enetic gain for fertility estimated breeding values in Holstein breed. The Jersey breed showed a decrease or no change in fertility rates for cows, while there was an increase in genetic gain in Jersey bulls during the post-genomic evaluation period of 2013–2017.

15.10 GS for Fertility in Buffalo

In buffaloes, there is only one report till date for genomic evaluation of reproductive traits (de Araujo Neto et al. 2020). One of the key reasons for this was the absence of a completely annotated genome. Although the buffalo genome was available from 2011 (Tantia et al. 2011), the annotation and updating were completed by 2019 (Low et al. 2019; Mintoo et al. 2019). Apart from these well-structured reference population is lacking for most of the buffalo breeds. In developing countries, absence of pedigree information and phenotypic data are also hindering the process. A way forward for GS in buffaloes will be the multi-breed genomic evaluation approach (Liu et al. 2018; Abdel-Shafy et al. 2020; Shao et al. 2021).

15.11 Future Prospects for GS for Fertility Traits in Bovines

15.11.1 Inclusion of Novel Traits

The future GS for fertility traits will have more emphasis on inclusion of novel fertility phenotypes which has higher heritability and can more comprehensively describe the biological process of reproduction.

The concentration of Anti-Mullerian Hormone (AMH) in blood is one of such traits with high heritability (Nawaz et al. 2018). The circulating AMH concentration is reported to be positively associated with the amount of antral follicles in ovary and extent of the ovarian reserve (Ireland et al. 2008). AMH can be used to determine ovarian progesterone, androgen production, herd longevity, pregnancy rate, and embryo production in dairy cows (Ireland et al. 2009; Mossa et al. 2010; Monniaux et al. 2010; Guerreiro et al. 2014; Ribeiro et al. 2014; Jimenez-Krassel et al. 2015). The Anti-Mullerian Hormone (AMH) concentration in blood was found to be highly variable among individuals and highly repeatable within animals (Ireland et al. 2008, 2011).

Other endocrine derived novel fertility phenotypes focusing on the milk progesterone levels have been suggested. The most studied one is the commencement of luteal activity (CLA) which is defined as the time elapsed (calculated as the number of days) postpartum to the initiation of luteal activity as pronounced by the rise in progesterone level in milk. The commencement of luteal activity postpartum has

been found to have a higher heritability (Darwash et al. 1997a; Petersson et al. 2007; Nyman et al. 2014). The cows which return to cyclic activity in postpartum early are associated with shortened calving to conception interval, improved conception rate and reduced services per conception (Darwash et al. 1997b). There are many other novel fertility phenotypes derived from the milk progesterone measurements like length of first luteal phase, occurrence of delayed luteolysis, proportion of samples with luteal activity, interluteal interval, prolonged luteal phase, and delayed ovulation (Royal et al. 2002; Nyman et al. 2014; Tenghe et al. 2015; Sorg et al. 2017). The advancement in farm automation technologies has allowed the in-line systems to instantly measure progesterone levels in milk making it a easy and cheap trait to record (Friggens et al. 2008).

15.11.2 GS Along with Assisted Reproductive Technologies

The use of GS in assisted reproductive technologies including ovum pick up and in vitro production of embryos coupled with embryo sexing and somatic cell nuclear transfer can lead to faster propagation of high genetic merit animals (Kadarmideen et al. 2018). In Nellore cattle, it was reported that incorporating GS in addition to the use of in vitro fertilization will result in 79% more yearly increase in genetic gain as compared to the normal situation (Carvalheiro 2014).

One of the promising technologies which has abundant potential to alter the intensity and direction of selection is the genome editing. In genome editing, nucleases are used to edit the genome to introduce desirable genetic variants. This technology can be incorporated with the assisted reproductive technologies as a method of introgression of desirable alleles.

15.11.3 Screening for Anti-Fertility Haplotypes

The GS leads to use of few outstanding sires in large scale. This warrants a constant vigil and surveillance so that undesirable genetic effects will not get spread through the populations. Currently, 14 lethal haplotypes related to fertility have been traced in the United States dairy breed genomic evaluation system which consists of Jersey, Brown Swiss Holsteins and Ayrshire breeds (Cole et al. 2015). Fritz et al. (2013) reported haplotypes associated with reductions in fertility with respect to conception rate in both heifers and adult cows in European dairy breeds. Haplotypes associated with reduction in fertility were also reported in Nordic Holstein and Fleckvieh cattle (Sahana et al. 2013; Pausch et al. 2015). A summary of haplotypes responsible for embryonic death is summarized in Table 15.3.

Table 15.3 Haplotypes responsible for embryonic death in cattle

Breed	Haplotype	BTA	Reference
Holstein (France)	HCD	11	Fritz et al. (2013)
	BY	21	
	HH1	5	
	HH2	1	
	HH3	8	
	HH4	1	
	HH5/HH6	3	
	HH13	18	
Montbeliarde	MH1	19	
	MH2	29	
	MH3	2	
	MH5	6	
	MH6	7	
	MH8	13	
Normande	NH1	24	
	NH2	1	
	NH5	7	
	NH6	15	
Fleckvieh	FH1	1	Pausch et al. (2015)
	FH2	1	
	FH3	10	
	FH4	12	
Ayrshire	AH1	17	Cole et al. (2015)
	AH2	3	
Brown Swiss	BH1	7	
	BH2	19	
Holstein (US)	HH0	21	
	HH1	5	
	HH2	1	
	HH3	8	
	HH4	1	
	HH5	9	
	HHC	3	
Jersey	JH1	15	
	JH2	26	

15.12 Conclusion

GS has led to improved genetic gain in bovine fertility traits. In future, there will be more emphasis on genomic prediction for novel fertility phenotypes and male fertility traits in dairy cattle and buffalo.

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Prospects of Stem Cells in Fertility Management in Bovine

16

Yasotha Thirupathi and Vikash Chandra

Abstract

High reproductive efficiency in dairy herd depends upon good farm management. Well managed dairies are utilizing advanced technologies for the improvement of fertility in bovines. In vitro fertilization (IVF) and embryo transfer have been proven as significant tool to increase the fertility in bovines. Apart from this, sometimes cows also get infertility problems like endometriosis, anoestrus, anovulation, early embryonic mortality, etc. Though traditional treatments are in place for the management of such problems, these have been found not so helpful in treating such disorders. In recent past, stem cell research has gathered significant attraction for treating reproductive disorders and improving reproductive efficiency in bovines. Spermatogonial stem cells have been successfully derived from testes, and both male and female gametes have been derived from induced pluripotent stem cells in mice. Similarly, oogonia like cells have been reported from ovarian cortex which indicates enormous use of proven cows. Most over, mesenchymal stem cells derived from different type of tissues offer great promise in the management of reproductive disorders and improvement of reproductive efficiency in bovine.

Keywords

Stem cells · Fertility management · Bovine · Conditioned media

Y. Thirupathi · V. Chandra (✉)
Division of Physiology and Climatology, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

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16.1 Introduction

Fertility plays a critical role in determination of the lifetime performance of a cow. As per the normal convention, a cow should yield a calf every year to be considered as fertile and prolific breeder. A healthy fertile animal possesses functional ovaries, exhibits normal oestrus behavior, mate, successful implantation, sustain the embryo throughout the gestation, giving birth of a calf, resume the cyclicity and restore the uterine function. The inherent fertility characteristics of an animal can be disturbed by multiple ways, including the nutrition, management, infectious and non-infectious cause. Infertility in which the animal experiences the temporary disturbance in normal reproductive function results in delayed or irregular production of a calf a year. All around the globe, great economic loss has been encountered due to fertility-related problems in livestock especially in bovine. Normal infertility treatment regimen follows the hormonal therapy and assisted reproductive techniques based on the underlying condition of the ailing animal.

Recently, stem cell technology has been revolutionized the field of regenerative therapy including human and livestock species owing to their special characteristics like high pluripotency, multilineage differentiation, immunomodulatory potential, chemotactic mechanism, etc. (Chandra et al. 2021). Stem cells can be obtained from wide range of sources like blastocysts, fetal tissues, and adult tissues and according to the origin, they have different characteristics (Fig. 16.1) (Chandra et al. 2021). Apart from these sources, adult somatic cells can be converted into embryonic cell type stem cells called induced pluripotent stem cells (iPSCs). Pluripotent stem cells have the ability to differentiate into all three germ layers (Evans and Kaufman 1981), whereas the multipotent adult stem cells can differentiate into few specialized cell types. Embryonic stem cells and iPSCs being highly immunogenic have limited scope in regenerative therapy while mesenchymal stem cells (MSCs) being very less immunogenic (due to immunomodulatory behavior) bear a great scope in regenerative therapy both in human and livestock species (Gugjoo et al. 2018; Bhat et al. 2019). MSCs do regeneration of damaged tissues mainly through three mechanisms: transdifferentiation into desired types of cells, immunomodulation, and secretion of

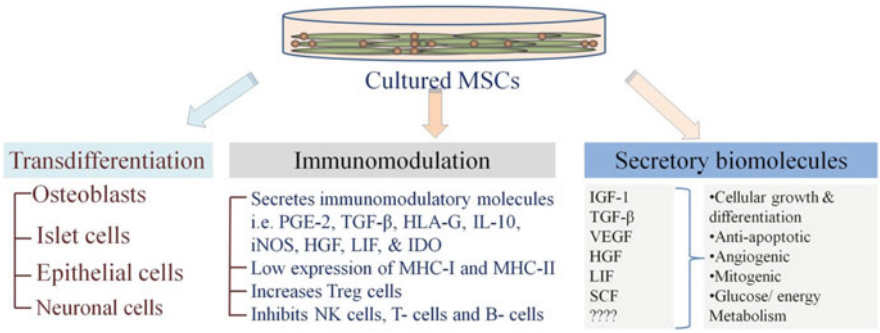

Fig. 16.1 Types of stem cells, origin and their characteristics

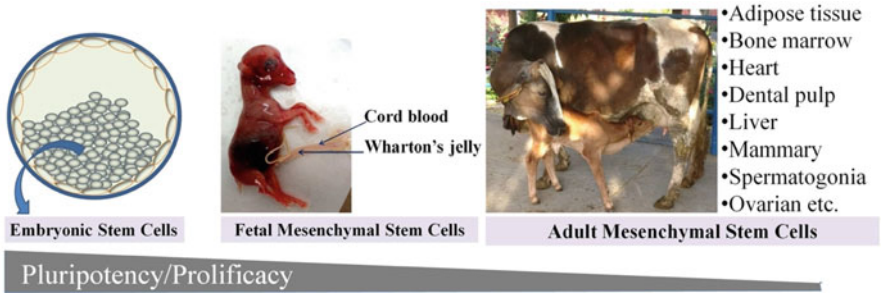

Fig. 16.2 Mechanism of action of mesenchymal stem cells in regeneration of tissues

important biomolecules (growth factors, cytokines, hormones, etc.) (Fig. 16.2). Recent past research demonstrated that the major mechanism of action of MSCs is through their secretory property. Use of MSCs- conditioned media (CM) in preclinical and clinical trials have proved that it has enormous scope in regenerative therapy (Ansari et al. 2013; Joseph et al. 2020).

These special properties of MSCs like regeneration potential, paracrine secretion of growth factors, enzymes and cytokines, immunomodulatory behavior have great prospects in treating infertility problems in bovine.

16.2 Embryonic Stem Cells

Embryonic stem cells are derived from the pre-implantation ICM (inner cell mass) of the dividing blastocyst and possess the capability for unlimited division and differentiation into cell types of all three germ layers. ESCs were generated in species like mice (Evans and Kaufman 1981), humans (Thomson et al. 1998), and cattle (Bogliotti et al. 2018). Stable ESC lines derived from the bovine can solve the complex mysteries of the early developmental process and pave the path for their efficient utilization in biotechnological intervention to improve reproduction efficiency. But there is still a considerable challenge persisting in generating stable ESC lines from cattle due to their restricted proliferation capacity and failure to exhibit pluripotent markers after long-term passages (Soto and Ross 2016; Kim et al. 2017). Primordial germ cells were derived from ESCs in human and mice, which further underwent meiosis to produce male and female gametes (Kehler et al. 2005). hESC-derived mesenchymal stem cells restored the ovarian function in the premature ovarian failure model in mice. The rate of ovulation and blastocyst formation were significantly improved in the treatment group (Yoon et al. 2020). hESC-derived endometrium-like cells were successfully recovered the structure and function of the uterine horn in a rat model of severe uterine damage (Song et al. 2015). The embryonic stem cells have a great potential in treatment of infertility in cattle.

16.3 Induced Pluripotent Stem Cells (iPSCs)

Adult somatic cells contain the dormant pluripotent genes, and their circulatory networks are again brought back to the pluripotent state by the ectopic introduction of transcription factors in reprogramming. Initially, the reprogramming approach was demonstrated by John Gurdon (1962) in frog and later by Takahashi and Yamanaka (2006) in mice. Considerable progress has been achieved in gamete generation from iPSC cells. Primordial germ cell-like cells (PGC-LC) were derived from iPSCs in mice (Imamura et al. 2010) and humans (Park et al. 2009). Among the livestock species, the porcine model is considered ideal for conducting clinical trials for human diseases as their physiology is similar to humans. In a study, PGC-LCs have been derived from piPSCs in vitro, and their developmental potential was analyzed by in vivo transplantation in mice seminiferous tubules that lack endogenous germ cells. The transplanted cells were successfully differentiated into spermatogonial stem cell-like cells (SSCLCs) and also expressed the key germline markers (Wang et al. 2016). biPSCs developed from bovine were induced into epiblast-like cells (EpiLCs) and iPGCs with the higher expression of H19, DNMT1, and DNMT3B genes indicating the process of epigenetic reprogramming (Bressan et al. 2018). Female gamete oocytes in metaphase II were developed from the iPSCs in mice in vitro in a three-step process and produced live fertile young ones of male and female (Hikabe et al. 2016). Male germ cells from spermatogonia to round spermatids were derived from iPSC-derived spermatogonial stem cells in mice (Zhu et al. 2012).

16.4 Adult Stem Cells

16.4.1 Spermatogonial Stem Cells

Spermatogenesis is a physiological process in males to produce haploid spermatozoa from the spermatogonial stem cells (SSCs) residing in seminiferous tubules. SSCs derived from testes maintain long-term availability of sperms throughout the life of a male (Kanatsu-Shinohara et al. 2008). Desirable elite characteristics of cattle can be disseminated by transplantation of adult gonadal stem cell population. Mouse SSCs were transplanted into seminiferous tubules of germline-depleted males and observed the resumption of sperm production (Kanatsu- Shinohara et al. 2003). Likewise, infertile non-human primates were transplanted with the allogenic and autogenic SSCs and found functional donor spermatogenesis (Hermann et al. 2012). Xenogeneic transplantation of SSCs from bull to mice testes suggested the initial colonization but fails to differentiate beyond the stage of spermatogonial cell proliferation (Dobrinski et al. 2000; Oatley 2018). Whereas, a complete regeneration of spermatogenesis has been observed in autologous transplantation of bovine SSCs (Izadyar et al. 2003). The benefits of improved in vitro culture conditions for maintaining bovine SSCs have been reported in several studies (Oatley 2018; Aponte et al. 2008). The advent of CRISPR/Cas9 editing allowed the generation

of germline ablated male cattle by knocking out the NANOS2 gene. Subsequent transplantation of SST with an allogenic donor resulted in sustained donor-derived spermatogenesis (Ciccarelli et al. 2020). Aforesaid studies indicate the potential opportunity of SSCs in treating male infertility in cattle.

16.4.2 Ovarian Stem Cells

The myth that the females carry only a finite number of germ cells in the ovary is a long-standing debate. However, oogonial stem cells (OSCs) have been isolated from the bovine ovarian cortex and were positive for the presence of VASA, a germ line-specific protein (Dunlop et al. 2014). The germline stem cells, i.e., PGCs and oocyte-like cells (OLCs) have been isolated from bovine ovaries and found to possess the normal morphological features: higher VASA expression and increased expression of specific mRNAs (de Souza et al. 2017). The presence of ovarian stem cells is confirmed by scraping the ovarian cortex samples in human, and existence of two populations of stem cells, viz. very small embryonic-like stem cells (VSELs) and ovarian stem cells has been reported (Bhartiya and Sharma 2020). More studies regarding the functional evaluation of OSC-derived PCGs and OLCs in bovine are highly warranted in the future studies.

16.4.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are better known for their immunomodulatory function as well as their transdifferentiation ability. So, this regenerative and immunomodulatory function of the mesenchymal stem cells can be explored for the treatment of infertility in bovine. In species like equine, the potential of MSC therapy on different long-standing reproductive problems such as endometritis, persistent breeding induced endometritis, endometriosis, ovarian and testicular diseases has been studied (Cequier et al. 2021). The modulatory potential of MSCs has been evaluated in in vitro embryo production (IVEP), and the authors reported improvement in embryo production rate when co-culturing with bovine embryos in a study (Miranda et al. 2016). Intraovarian injection of MSCs improved the in vitro embryo production and also the oocyte yield in the bovine infertility model. Local injection of MSC after OPU has been found to restore the fertility cows but to treat cows with disorders of cystic ovarian and chronic ovarian lesions (Malard et al. 2020). However, Peng and co-workers showed the efficacy of cattle placental derived MSCs (CPSCs) for the treatment of ovarian follicular cyst (OFC) in Holstein Friesian cattle. Injection of CPSCs in the ovary showed prominent estrus with a higher oocyte recovery and inseminate conception rate in HF cattle (Peng et al. 2020). Angiogenesis, paracrine secretion, and immunomodulation properties of the MSCs hold promising potential in the treatment of infertility cows. In a study, bovine adipose-derived MSCs delivered via the intravenous route (IV) showed better healing of endometrium in the metritis cow model. The reduction of the turbidity of cervical

fluid and mucus in the IV group proved the therapeutic value of MSCs in treating cow infertility (Singh et al. 2019). The therapeutic effect of MSCs and MSC-derived exosomes on endometrial damage and restoring fertility has already been studied in the rodent model (Zhang et al. 2018; Xin et al. 2019; Xin et al. 2020). However, there is no report of bovine MSCs used for the repair of damaged endometrium, which leaves us a broad scope in exploring the potential of MSCs in the therapy of uterine disorders in bovine.

Endometrial mesenchymal stem cells (eMSCs) research in livestock has been scarce, but a study in bovine demonstrated the potential role of eMSCs in modulation, migration, and homing during the uterine inflammatory condition in endometritis. When the eMSCs were challenged with PGE2, uterine inflammatory mediator, in vitro, the normal eMSC transcriptomic profile has been altered (Lara et al. 2017). The therapeutic value of amniotic derived MSCs (ADMSCs) from bovine to treat bilateral ovarian dystrophy was studied by Chang and co-workers, and they found that the group treated with an intraovarian injection of ADMSCs showed high oestrus (50%) and pregnancy rates (25%) than the control group (Chang et al. 2018). Most of the research findings mentioned above strongly suggest the potential utilization of MSCs for restoring fertility in bovine.

16.4.4 MSCs Conditioned Media for Quality Embryo Production In Vitro

Major cause of infertility in humans and domestic animals is implantation failure. Embryonic mortality is a major cause of economic loss in dairy production systems. The incidence of embryonic mortality in naturally mated bovine is approximately 20% (Campanile and Neglia 2007). Bi-directional embryo-maternal communication is required for proper embryonic development and maintaining an adequate uterine environment. In vitro embryo production and transfer technology is a vast growing field which has great potential in enhancing reproductive efficiency in cattle and buffalo (Viana et al. 2018; Pandey et al. 2021). In repeat breeders, ET following AI is proved beneficial in improving conception rate. But the in vitro quality embryo production is the major limitation for the vast application of this technology (Chandra and Sharma 2020). In the mammalian reproductive tract, the oviduct secretes various kinds of growth factors and cytokines, which play an essential role for the development of initial stages of pre-implantation. Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) have been reported to secrete several such biomolecules and supplementation of its conditioned media (CM) in culture media during in vitro embryo culture improved quality blastocyst production significantly (Bhardwaj et al. 2016).

16.5 Conclusion

Fertility-related problems in bovine are routinely treated with external hormonal supplements and nutritional management although the fundamental assisted reproductive techniques offer a substantial therapeutic value. The versatile stem cell sources and their characterization have been sufficiently documented in a vast number of research findings. The therapeutic utility of stem cells for infertility in different animal species has also been verified in a multitude of research finding, but there are still scanty reports are available in cattle. So, it can be advocated that stem cell therapy offers a potential future prospect for the management of reproductive disorders and enhancement of reproductive efficiency in cattle.

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Conclusion

17

A. Kumaresan and A. K. Srivastava

Abstract

In bovines, traditional selection schemes selected animals predominantly for milk yield often at the expense of other traits including fertility; therefore, over a period of time, the fertility parameters showed a declining trend. Reduced reproductive efficiency due to altered reproductive physiology in high producing bovines requires application of reproductive technologies to restore the fertility. During the recent past, new reproductive technologies have been developed, and old technologies were further refined to improve their efficiency. These technologies including multiple ovulation and embryo transfer, ovum pick up and in vitro embryo production, semen sexing, gamete cryopreservation, fertility biomarkers, and controlled breeding protocols have immense role in improving reproductive efficiency in bovines. With the advancements in analytical tools and scientific methods, now, it is possible for further fine tuning of the potential technologies to improve fertility in bovines.

Keywords

Reproductive technologies · Recent advances · Bovine fertility

The advent of intensification in bovine production has undoubtedly posed several challenges to the entrepreneurs; the most important one is decline in fertility of

A. Kumaresan (✉)
Theriogenology Laboratory, Southern Regional Station of ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India
e-mail: A.Kumaresan@icar.gov.in

A. K. Srivastava
Agricultural Scientists Recruitment Board, New Delhi, India

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modern bovines. Since fertility is a multi-factorial trait and its deterioration has been caused by a network of genetic, environmental, and managerial factors, and their complex interactions make it difficult to determine the exact reason for this decline. With the growing concern over declining reproduction efficiency, the pragmatic approach could be the use of biotechnological options for identification of etiology and restoring the fertility. In this direction, several approaches have been employed to augment fertility in infertile animals or to reduce the magnitude of high-production associated decline in fertility. Reproductive biotechnology has taken a gigantic leap in the last few decades and powerful reproductive technologies such as oestrus induction, oestrus and ovulation synchronization, superovulation, artificial insemination, embryo collection and transfer, in vitro embryo production, gamete cryopreservation, a variety of embryo micromanipulation procedures, and nuclear transfer have been developed. The first generation of reproduction biotechnology that has played an unequivocal role in genetic improvement and production enhancement, at least in large animals, is artificial insemination. The second generation of reproductive biotechnology, multiple ovulation and embryo transfer has been in use for more than fifty years. The third generation of reproduction biotechnology, in vitro fertilization, is more recent and in use from late 1980s. This technology allows collecting the oocytes very easily from the slaughterhouse as well as collecting the oocytes from live animals through "ovum pick up." In countries like India, where cow slaughter is banned, ovum pick up offers immense scope for obtaining oocytes from elite live cows for in vitro embryo production and faster propagation of elite germplasm. The fourth generation of reproduction biotechnology, nuclear transfer and transgenesis, is the latest development and mostly restricted to laboratories or used in very small scale. The fourth generation of reproductive biotechnologies are yet to be accepted widely because of various reasons including ethical concerns, risks associated with food safety and concern about the expected benefit from use of these technologies. It will take sometime to realize the potential benefits or risks of these technologies. Other techniques associated with reproductive technologies have an immense role in success of the purpose of using these technologies including sperm sexing, biomarkers for fertility prediction, gamete cryopreservation, quality improvement of frozen spermatozoa, and fertility improvement protocols. Regular use of ultrasonography for monitoring the reproductive physiology of bovines would help in identification of problematic animals at an early stage. In large scale commercial herds, infrared thermography can be used for precision and remote monitoring of reproductive events.

Among the several technologies developed, reproduction biotechnologies are excellent model of technology transfer between the research laboratory and farms because the adaption of these technologies has been exemplary. The infrastructure and high cost associated with some of the technologies and inadequate expertise and inaccessibility of these techniques to the stake holders are the factors preventing the harness of full potential of these techniques. In several countries, use of these techniques is in the process but still the developments in this direction are in its infancy and yet to be explored in large commercial scale. Nevertheless, the time has come up to realize the full potential of these technologies for their successful

application, wherever required, for multiplication of superior germplasm that is in great demand nowadays. Innovative research in animal reproduction techniques is fundamental to the future bovine industry. Additional robustness can be brought in the identified technologies to suit the management and socio-economic conditions for effective implementation of technologies under a given situation.