A. Kumaresan

A. K. Srivastava   *Editors*

Current Concepts in Bovine Reproduction

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Preface

Bovine production largely depends upon reproduction. Successful reproduction with optimum fertility is the major factor determining the farm efficiency and profitability in bovines. In the recent past, several studies expressed concerns over the bovine fertility, which showed a declining trend during last few decades. Over the period, the dairy bovines have been selected for high performance; milk productivity has steadily increased due to genetic improvement combined with better nutrition and management. On the other hand, selection of animals for high milk production has also changed the metabolic adaptation and reproductive physiology of animals, leading to decreased reproductive efficiency. Delayed sexual maturity, extended calving to conception interval, and poor conception rate continue to remain as major problems in considerable population of animals leading to reduced lifetime production. This situation calls for a thorough understanding of the basics and advanced concepts of physio-pathology of bovine reproduction so that they can be applied on animals to improve upon the reproductive efficiency.

Due to advancements in science and scientific methods, during recent times, several developments have taken place in fertility management of farm animals. Recent concepts in bovine reproduction have opened up new avenues for manipulating the reproductive process both in vitro and in vivo for improving their reproductive efficiency. Updating knowledge on the recent developments in fertility management would help in wider dissemination and application of frontier technologies for improving the reproductive efficiency of dairy bovines. There has been quite a number of developments in the area of sperm sexing, biomarkers for fertility prediction, gamete cryopreservation, quality improvement of frozen spermatozoa, and fertility improvement protocols. However, the recent developments are scattered in the form of research papers and information on the established facts are not readily available to the end users. Therefore, in this book, we attempted to provide 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 this book, current concepts in the area of bovine reproduction including the targets for reproductive efficiency, reproductive cyclicity, regulation of reproductive events, controlled breeding programmes, uterine infection, semen cryopreservation and quality assessment, and epigenetic bearing on reproduction are covered. We

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strongly believe that the book will be immensely useful to the students, researchers, and bovine farm managers in updating their knowledge on bovine reproduction.

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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 post-doc 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 molecules, 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 Young Researcher Award in 2015, and Outstanding Research Faculty Award by Career 360 in 2018. He is a member of many scientific societies and organizations, fellow of the National Academy of Agricultural Sciences, the National Academy of Dairy Sciences, and the 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 and Director Resident Instructions and 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 post-doc 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 the 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 the National Academy of Sciences, fellow of the National Academy of Agricultural Sciences, fellow of the National Academy of Dairy Sciences, and fellow of the 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 1987–1988 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 "Lifetime Achievement Award" by the Indian Society for the Study of Reproduction and Fertility, the Society for Community Mobilization for Sustainable Development, and the 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, and Dr. Rao Memorial Oration Award, Dr. V. Kurien Memorial Oration Award, and Prof. S. Kannaiyan Memorial Award-2019, by the 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

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Abstract

Over a period of time, there has been a decline in fertility of bovines. Probably, intense selection for milk production coupled with a variety of physiological and management factors could be attributed for the decline in fertility. On the other hand, the demand for milk and meat is increasing. This situation warrants a thorough understanding of underlying etiology for reduced fertility so that the fertility can be restored in infertile/subfertile bovines. This book provides a 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 estrus detection, scope for improving reproduction efficiency using timed breeding protocols, emerging concepts on epigenetic bearing on fertility, and semen cryopreservation and quality control.

Keywords

Bovine · Fertility · Nutrition · Controlled breeding · Semen quality

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Reproduction is a biological process through which continuity of the species is ensured. Optimum reproduction efficiency is the basic prerequisite for obtaining success in bovine farming. Before the period when commercial bovine rearing started, the animals were in their natural way of living as a herd comprising of males and females wherein the potential males identified the animals in estrus and served them. Although the reproductive efficiency in such herds is not clearly quantified, it is true that a majority of the reproductive disorders and inefficiencies observed currently in commercial scale bovine farming did not either exist or were documented. In the process of commercialization, the males and females were separately reared under different locations and the females were served artificially using the semen collected from the potential males. Additionally, intense selection was applied for either milk (in dairy bovine) or meat (in beef bovine) production, leaving aside the reproduction, resulting in altered reproductive physiology in high-yielding bovines. Moreover, the heritability of reproductive parameters is low to moderate. All these collectively resulted in reduced reproductive efficiency in bovines.

It is often said that "reproduction is a luxurious phenomenon" for an individual. Primarily, a cow gives higher priority to her own maintenance followed by growth, lactation, fetal growth, and then breeding. Therefore, to achieve high reproduction efficiency, all the basic and necessary needs of the animal should be fulfilled, and when all her body requirements are fulfilled, she diverts her energy toward reproduction. To obtain maximum reproductive efficiency in bovines, matching of genotypes to proper production environment in terms of appropriate husbandry practices is very much essential. The aim should be to ensure that the intervals from calving to conception are short and the rates of conception to natural or artificial breeding are high so that the calving intervals are short and a greater number of calves and lactations could be achieved in the reproductive lifetime of a cow. One of the major reasons for reduced lifetime milk production by an individual dairy animal is transient loss of fertility or infertility. The causes of infertility in dairy animals are many and can be complex. They relate to follicle development and maturation, onset of estrus, successful coitus/insemination, ovulation, fertilization, implantation, the development and delivery of the normal fetus and its membranes, proper uterine involution and cleansing, resumption of ovarian cyclicity, and estrus expression. Anything interfering with the routines of this cycle, such as diseases, poor nutrition, inadequate herd management, hereditary and congenital factors, hormonal disturbances, or environmental changes, makes the animal infertile, if only temporary in occurrence.

In the recent past, because of advancements in techniques for understanding the molecular events controlling the fertility/infertility, several new concepts have been evolved in bovine fertility management. Updating knowledge on the recent concepts in fertility management is essential for their application in improving the reproductive efficiency of bovines. It is against this backdrop, this book is brought up with the aim to appraise the readers about the recent developments accumulated in the area of fertility management and to provide an updated knowledge on current concepts for improving reproduction efficiency in male and female bovines.

The first chapter introduces the global trends in reproductive efficiency of bovines, the plausible reasons for declining fertility in bovines, benchmarks for bovine reproduction assessment, long-term and short-term approaches for fertility enhancement, and the developments in controlled breeding programs for improving individual bovine fertility. Because onset of puberty and subsequent reproductive processes are the consequences of interactions of the hypothalamo-pituitary-gonadal axis mainly through the stimulation of gonadotropin-releasing hormone (GnRH), understanding the GnRH stimulators is essential to augment fertility. Accordingly, the second chapter exclusively discuss on the role of kisspeptins, the most potent GnRH-secretagogue in mammalian species, in bovine reproduction. This chapter provides an overview of our current knowledge on kisspeptin-kisspeptin receptor system with a special focus on the role of kisspeptin-KiSS1/R system in bovine reproduction. As indicated earlier, the cow's priority for reproduction depends upon how sound is her nutritional status; only when she is in adequate energy balance one can expect her to reproduce efficiently. A thorough understanding of the nutritional requirements of bovines and about the nutrients required for efficient reproduction, their function, level of feeding, and feeding strategies is important to achieve optimum reproductive efficiency. Therefore, the third and fourth chapters provide insights on the role of metabolic hormones and nutritional strategies to improve reproductive efficiency in cattle and buffaloes including low-cost feed formulations using the available resources.

Proper estrus detection is one of the major bottlenecks in bovine farms; generally estrus in bovines is detected either manually or using automated aids; however, their efficiency varies with the scale of operation. Salivary fern pattern is used in human beings to precisely determine the fertile period. Very recently, the concept of use of salivary fern pattern or micromolecules as a noninvasive tool for estrus in bovines has gained momentum. In this regard, a chapter has been dedicated to detail the use of salivary fern pattern and salivary molecules as an aid for estrus detection in bovines. Identification of animals at estrus and inseminating them at right time does not guarantee conception unless the cow has a healthy uterine environment. Knowledge about the expression and regulation of endometrial Toll-like receptors (TLRs) during the reproductive cycle is important to understand the clearance of uterine infections in the bovine. The sixth chapter details the expression pattern of TLRs in the endometrium, regulation by phases of the estrous cycle, and their significance in the uterine health. Upon successful fertilization, the developing embryo must provide a signal to the mother so that she recognizes the presence of the embryo in the uterus and maintain the pregnancy; this process is called as maternal recognition of pregnancy (MRP). Understanding the mechanism of MRP will pave the way forward to develop a therapy intended to rescue the pregnancy losses by improving embryo survival. The seventh chapter provides a deep understanding and recent concepts on MRP in bovines.

Difficulties in estrus detection can be overcome by using timed artificial insemination of cows. During recent years, there have been a lot of advancements in understanding the role of hormones, which facilitated development of different protocols for either timed breeding or estrous synchronization. The eight chapter

elaborately discusses the physiology of estrous cycle and approaches for estrous synchronization and controlled breeding besides explaining different protocols and their advantages and disadvantages. Among the several reasons for postpartum infertility, uterine infection alone accounts for more than 30%. A better understanding of the pathogens causing uterine infection, host immunity, impact of uterine infection on production and reproduction, and identifying risk factors associated with development of uterine infection would help in developing effective preventive and therapeutic strategies for downsizing the incidence of uterine infection in dairy animals to ensure increased production and profitability besides supply of safe milk and milk products to the consumers. Accordingly, the ninth and tenth chapters are dedicated to herd health concept for understanding and minimizing the incidence of uterine infection in bovines besides detailing the effective preventive and therapeutic measures including alternate approaches to the use of antimicrobials in treatment of uterine infection. The molecular advancements in understanding the development of uterine infection or elimination of microbial contaminants from the uterus are also detailed in these chapters.

Currently, cryopreserved semen is used for artificial breeding of bovines. Procedures involved in sperm cryopreservation including extension, cooling, freezing, and thawing reduce the sperm fertility: approximately 50% of spermatozoa become immotile during the process. Therefore, bovine sperm cryopreservation protocol needs improvement for harvesting higher proportion of viable and fertile sperm cells after thawing. The tenth chapter details the advancements in extenders, process of cryopreservation, and the measures for minimizing the cryopreservation associated damages to spermatozoa. Because of the immense contribution of buffaloes in terms of milk and meat, and uniqueness of spermatozoa that differs in several aspects from that of cattle bulls, a special chapter on buffalo semen cryopreservation is also included in the book. This chapter elaborates the factors affecting cryo-survivability of buffalo spermatozoa and supplementation of the freezing medium with novel cryoprotectants, antioxidants, and other new components such as proteins or nanoparticles for reducing the cryodamage to spermatozoa.

The role of male fertility is immense in bovines, because semen from one bull is used to artificially breed several thousands of cows. Currently, the breeding bulls are selected based on breeding soundness evaluation (BSE) that assesses the mating and semen production ability of the bull. The traditional semen quality assays performed as a part of BSE do not reflect the sperm fertilizing potential. Therefore, a lot of researches have been carried out to develop tests that have a good relationship with sperm fertility. In the recent past, along with advancements in high-throughput sperm analysis, techniques such as flow cytometry, several sperm function tests have been found to have high correlation with fertility. A battery of tests needs to be carried out to arrive at prediction of fertility with fair accuracy. Chapter 13 provides a deep insight into the advances in sperm quality assessment and suggests the tests that have very high potential to be used in fertility prediction. The last two chapters are dedicated to improving sperm fertility using proteins associated with sperm fertility and sperm survival during its tortuous journey in the female reproductive tract. Chapter 14 introduces a novel concept of coating sperm with β-defensins to protect

sperm from maternal immunologic aggression. Finally, the 15th chapter deals with the emerging concept of epigenetic bearing on fertility including a comprehensive understanding of mechanisms of epigenetic modifications in germ cells and attempts to correlate the implications of DNA methylation during epigenetic reprogramming, epigenetic potential of the nuclear proteome, and abnormal protamine expression with the fertility.

Reproduction Efficiency in Dairy Bovine: Trends and Targets

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Abstract

Reproductive efficiency is the major factor determining productivity in dairy bovine. In spite of several technological advancements, poor fertility of dairy bovine continues to be a major factor limiting the profitability of dairying. Fertility is a multifactorial trait influenced by genetics, environment and management, and their complex interactions, making it difficult to determine the precise reason for poor fertility. Over the period, the dairy bovines have been selected for high performance; milk productivity has steadily increased due to genetic improvement combined with better nutrition and management. On the other hand, selection of animals for high milk production has also changed the metabolic adaptation and reproductive physiology of animals, leading to decreased reproductive efficiency. Several researchers reported that greater milk production was associated with reduced reproductive performance in dairy cattle. However, an interesting fact is that declines in fertility are observed in the lactating cows rather than for the heifers, indicating that the impact of genetic selection for higher milk production on inherent fertility is likely to be modest. This offers scope for application of modern reproductive technologies coupled with environmental, managemental, and nutritional interventions to improve the reproductive efficiency in the dairy bovine. In this chapter, targets for dairy bovine reproductive efficiency, how far the set targets are achieved, the possible reasons for

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reduced fertility, and an overview of reproductive technologies for improving fertility are discussed.

2.1   Introduction

Dairy bovine productivity largely depends upon reproduction, which in turn is influenced by several factors including genetic, nutritional, hormonal, physio-pathological, and management practices (Pryce et al. 2004; Veerkamp and Beerda 2007; Löf et al. 2007). Reproductive disorders and infertility in dairy bovine pose serious economic loss to farmers by reducing returns and increasing veterinary expenses. The target for successful reproduction efficiency in dairy cows is to accomplish "a calf per cow per year" and in case of buffaloes, it is "a calf per buffalo cow in every 14–15 months." To achieve this target, a cow or buffalo should conceive within 85–115 days postpartum, which in turn requires that the animal should start cyclicity as early as 45 days and breeding should start by 60 days postpartum. But this is seldom achieved because of postpartum complications including prolonged acyclicity, uterine infection, and negative energy balance (Dobson et al. 2008; Lucy 2008; Sheldon et al. 2008).

Several studies have reported a decreasing trend in the fertility of dairy cows over a period of time. Trends of greater numbers of days open, services per conception, and extended calving intervals have been reported in dairy cows over the past 25–35 years. A report by the Swedish Dairy Association indicated that the calving interval of Swedish Holstein cattle increased to 13.6 months in 2012 from 12.5 months in 1985 (Swedish Dairy Association 2012). In general, high production levels selected for dairy herds have been reported as a reason for fertility decline in dairy cattle (Hansen 2000; LeBlanc 2010); however, a clear consensus it yet to be arrived. While several studies have shown clear negative genetic relationship between milk yield and fertility, others have shown there are high producing dairy animals with good fertility. Although declining dairy cow fertility is a concern for several countries across the globe, some of the highest yielding and most productive herds in some countries are able to maintain outstanding fertility. This clearly demonstrates that poor fertility is not an inevitable consequence of modern dairy systems (Arnott et al. 2015). Maintaining good fertility requires a perfect matching between genotypes and the production environment, along with appropriate management practices (Rauw and Gomez-Raya 2015). Therefore, it is possible to achieve high fertility in high yielding dairy bovine through proper management, nutrition, and application of appropriate reproductive technologies.

Universally, it is well recognized that reproductive technologies have revolutionized dairy production. Recent advancements in reproductive technologies facilitated manipulation of the reproductive process in dairy animals for improving

their reproductive efficiency. Reproduction technologies are considered to be good model of technology transfer between the laboratory and the farms, since the adaption of these technologies has been exemplary. In several countries, advanced reproduction technologies (e.g., ovum pick-up and in vitro embryo production) are routinely applied at grassroot level and the impact of these technologies on animal production and on the economic developments of farmers is very evident. On the other hand, in other countries, the first-generation reproduction technologies (For instance artificial insemination) are the only technologies available to the farmers and other frontier technologies are mostly restricted to laboratories. In these countries, requirement for high initial investment, infrastructure, and inadequate expertise are the few factors limiting the extensive use of these technologies. Nevertheless, the time has come up to realize the full potential of these technologies.

2.2 Dairy Reproduction Benchmarks

To achieve high reproduction efficiency in bovines, a thorough knowledge on indicators of reproductive efficiency, their application in day-to-day farm operations, and overall improvement of selected reproductive parameters are important. Few important reproductive efficiency parameters are defined here and the targets for the reproductive efficiency parameters are given in Table 2.1.

- *Conception rate:* Conception rate is defined as the proportion of inseminations leading to a pregnancy. It is the number of pregnant cows over total of services in a given period of time. Although it is commonly calculated on a monthly basis, it can be used for any defined group of inseminations.
- *Pregnancy rate:* It is the result of conception rate X the service rate. It measures how fast cows get pregnant every 21 days; therefore, it is measured in a time interval of 21 days. Service rate is the number of eligible cows served within a period of 21 days.

Table 2.1 Dairy cattle reproductive efficiency parameters and their targets (Compiled from different sources)

Parameter	Target
Conception rate at first artificial insemination	55–60%
Number of AIs required for conception	Less than 2.0
Calving interval	365–375 days
Calving to first service interval	60–65 days
Voluntary waiting period	45–55 days
Submission rate	70–80%
Heat detection rate	70–80%
Calving to conception interval	85–100 days
Days open	120 days
Nonreturn rate	>75%
Overall pregnancy rate	>65%
Culling rate due to reproductive failure	<8%

- *Calving interval:* Calving interval represents the number of days between two successive calvings. Calving interval includes the measure of return to cyclicity and conception; however, at times, it may be biased, because infertile cows are often subjected to early culling and hence do not recalve. At the herd level, the average calving interval, across a group of lactations, is referred to as the calving index, which fairly indicates the overall picture of fertility for an all-year calving herd.
- *Calving to first service interval:* Calving to first service interval is the number of days between the day of calving and the day of her first service postcalving. This interval is influenced by herd management, which needs to be considered in fertility evaluations. While calving to first service interval is a measure of return to postpartum cyclicity, it does not provide information about conception. Excessively long calving to first service intervals indicates considerable time delays and financial losses.
- *Voluntary waiting period:* The Voluntary Waiting Period is the period after calving when cows are intentionally not allowed for mating/serving. The Voluntary Waiting Period could have an influence on first service conception rates and lactation length.
- *Submission rate:* Submission Rate is a valuable measure for all herds. Submission Rate for a month is the percentage of cows inseminated at least once during that particular month of the mating period, which begins after completion of the Voluntary Waiting Period.
- *Heat detection rate:* Heat Detection Rate is the percentage of cows correctly identified in heat out of those cows eligible for heat in a defined period. It is one of the key indicators of estrus expression intensity and estrus detection efficiency.
- *Calving to conception interval:* It is the time interval between calving and the service that resulted in pregnancy. Calving to conception interval is simply a way to measure calving interval with a shorter time lag.
- *Days open:* Days open is the period between the date of calving and the date of last insemination that resulted in conception or culling or death. For cows that conceive, days open is equivalent to calving to conception interval. For those cows not conceiving, days open is the period from calving to culling or death, or the maximum interval from calving to insemination.
- *First service pregnancy rate:* This indicates the cows/heifers conceived at the first service itself. First service pregnancy rate is the percentage of cows/heifers that conceived out of the total number of first services given over a period or to a group of animals. First service pregnancy rate is one of the most valuable measures of dairy herd fertility.
- *Nonreturn rate:* The nonreturn rate is the proportion of inseminated cows that are not subsequently rebred within a specified time period. NR is the most commonly used measure for bull fertility evaluations and the commonly studied periods are 28 days (NR 28), 56 days (NR 56), or 90 days (NR 90) after mating/insemination.
- *Overall pregnancy rate:* It is the percentage of animals conceived out of the total number of services given to a group of animals or over a specified period.

- *Culling rate:* The culling rate is the proportion of cows removed from the herd during a defined period (usually 12 months) out of the total number of animals.

2.3   Is Fertility Really Declining in Dairy Animals?

Several studies originating from all over the globe reported a decrease in the fertility of dairy bovine. For instance, in the USA and England, it is reported that the first insemination conception rate has decreased by 0.45% and 1%, respectively, per year over a period of 20 years (Butler and Smith 1989; Royal et al. 2000a, b). On the other hand, few reports indicate some of the highest yielding and the most productive herds in the UK were able to maintain very good cow fertility. This clearly demonstrates that poor fertility performance is not an inevitable consequence in modern dairying. The reported changes in the fertility status of dairy bovine over a period of time are summarized in Table 2.2.

Both the negative relationship between milk production and reproduction, and the changes in the management practices to support high milk production, could explain the decline in dairy cattle fertility (Lucy 2007). Milk production and fertility are two economically important traits that are polygenic, affected by many genes and variants, with each gene having small effects on the observed phenotype (Snelling et al. 2013). During the past few decades, intense genetic selection has increased milk production potential of the cow, but also changed the reproductive physiology leading to a decrease in reproductive efficiency (Lucy 2001). But as on date, controversy exists among the researchers regarding the trend of dairy cattle fertility over few decades and also about the relationship between milk yield and fertility. While few report no decline in fertility, a majority reports indicate that the dairy cattle fertility is decreasing over a period of time. For instance, over the past 50 years, it has been reported that the percentage of estrus animals showing standing to be mounted activity has declined from 80% to 50% and the duration of estrus has declined from 15 h to 5 h. During the corresponding period, the first service conception rate declined from 70% to 50% (Dobson et al. 2008), while the days open and the number of services per conception has increased (Lucy 2001). Tracing back to the history of selection of dairy animals, one can evidently note that animals were selected mainly for milk production ability and reproduction parameters have not been given due importance. Based on the large datasets, a clear antagonistic relationship between milk production and reproduction has been reported in dairy cattle (Dematawewa and Berger 1998; Hansen 2000). On the other hand, few reports also indicate that the historical decrease in fertility has reached a nadir and started to improve (Crowe 2007; Norman et al. 2009); however, further studies involving large sample size are required to confirm this trend. In a review by LeBlanc (2010), it is urged to critically reanalyze the studies, which have made conclusions on the association of level of milk production with fertility based on incomplete or biased datasets. It is also indicated that improper management of high yielding dairy cows might have significantly contributed to the poor fertility rather than direct effects of genetics. A word of caution for the readers is that all these studies were conducted on

Table 2.2 Trends in dairy cow fertility reported in the literature

Country	Fertility parameter	Change in fertility	Reference
USA	Conception rate at first artificial insemination	Decreased by 0.45% per year	Beam and Butler (1999)
USA	Number of AIs required for conception	Increased from 1.75 to more than 3	Lucy (2001)
USA	Calving interval and conception rate	Deterioration in both measures (decrease in calving interval more pronounced)	Norman et al. (2009)
England	Conception rate at first artificial insemination	Decreased by 1% per year	Royal et al. (2000a, b)
UK	Calving interval, conception rate, and calving to first service interval	Improvement in calving interval and calving to first service interval; conception rate stable	Hanks and Kossaibati (2012)
The Netherlands	Conception rate at first artificial insemination	Decreased from 55.5% to 45.5%	Jorristma and Jorristma (2000)
Spain	Ovarian inactivity	Increased by 4.6%	Bousquet et al. (2004)
Ireland	Number of AIs required for conception	Increased from 1.54 to 1.75	Mee et al. (2004)
Ireland	Conception rate	Decreased from 64.9 to 57.1%	Mee et al. (2004)
France	Nonreturn rate	Decreased by 15%	Bousquet et al. (2004)
Canada	Nonreturn rate	Decreased from 69 to 67%	Van Doormall (2002)
Canada	Conception rate at first artificial insemination	Decreased from 44 to 39%	Bouchard and Du Tremblay (2003)
Canada	Conception rate at second artificial insemination	Decreased from 47 to 41%	Bouchard and Du Tremblay (2003)
Canada	Number of AIs per lactation	Increased by 0.48 per cow per lactation	Bouchard and Du Tremblay (2003)
Canada	Submission rate and conception rate	Relatively stable	LeBlanc (2010)
Sweden	Calving interval	Increased to 13.6 months from 12.5 months	Swedish Dairy Association (2012)
Sweden	Calving interval and calving to first service interval	Deterioration in both the parameters	Löf et al. (2007)
Norway	Calving interval and calving to first service interval	Relatively stable	Refsdal (2007)
Australia	Submission rate and conception rate	Slight deterioration	Morton (2011)

high yielding cows and the same may not be extrapolated to moderate or low yielding dairy animals. Based on the forgoing findings, it is logical to infer that intense selection for high milk production negatively impacted the physiology of cows leading to reduced likelihood of the pregnancy establishment; however, suitable modifications in management practices may help to improve fertility in high producing cows (Walsh et al. 2011).

Although the decline in reproductive efficiency of cows is chiefly related to changes in management, it is reasonable to attribute a portion of this decline to the males (DeJarnette et al. 2004). Several studies reported that infertility and subfertility in males costs significantly to dairy production (Larson and Miller 2000). Moreover, several scientific surveys on the dairy industry reported that fertility of bulls has also declined over a period of time (Cropp et al. 2005). Recently, the trend of subfertility in crossbred bulls belonging to three generation was studied and found an increasing trend as generation advances (Vijetha et al. 2014). The mean age at first semen collection increased by 66 days in sires compared to their grandsires. Also, the age at first semen freezing (AFSF) showed a linear increase from grand sire to son. In grand sires, the AFSF was 767.14 ± 25.82 days, while the AFSF in sires was 831.43 ± 31.17 days, which further increased to 871.25 ± 61.82 days in son. The semen production period (SPP) was higher in grand sires compared to sires and sons (Fig. 2.1). Similarly, the sons and sires produced higher percentage of poor ejaculates compared to grand sires. Although management could be a reason for the differences in AFSC, AFSF, and SPP among grand sires, sires, and sons, the data indicates a decreasing trend in quality semen production ability. When a breeding bull suffers from subfertility/infertility, it can have a significant effect on reproductive efficiency of cows, because semen from one male is used to breed several thousand of females. In a very recent review, it is reported that low-fertile bulls had altered testicular cytology indices, reproductive hormone concentrations, sperm functional attributes, and seminal plasma composition as compared to high-fertile bulls (Kumaresan et al. 2021).

2.4 How Increased Productivity Could Affect Reproductive Events?

Reduced reproduction efficiency in high yielding animals could be explained by the physiological changes associated with the increased milk production. For instance, in dairy cows, in addition to increased milk yield, greater herd size, modifications in housing conditions, and increase in do-it-yourself artificial insemination have all contributed to difficulties in achieving high fertility in high producing dairy cows (Rodriguez-Martinez et al. 2008). The possible ways by which higher milk production could be associated with impaired reproduction are indicated in Fig. 2.2. Reported physiological alterations in high milk producing cattle are indicated below.

- *Nutritional stress*: There is a rise in the energy demand in high milk-producing dairy cows. The increased energy demands could be met only partially by

Fig. 2.1 Trend in production
of quality semen by crossbred
breeding bulls. *AFSF* age at
first freezable semen
production, *ERR* ejaculate
rejection rate, *SPP* semen
production period
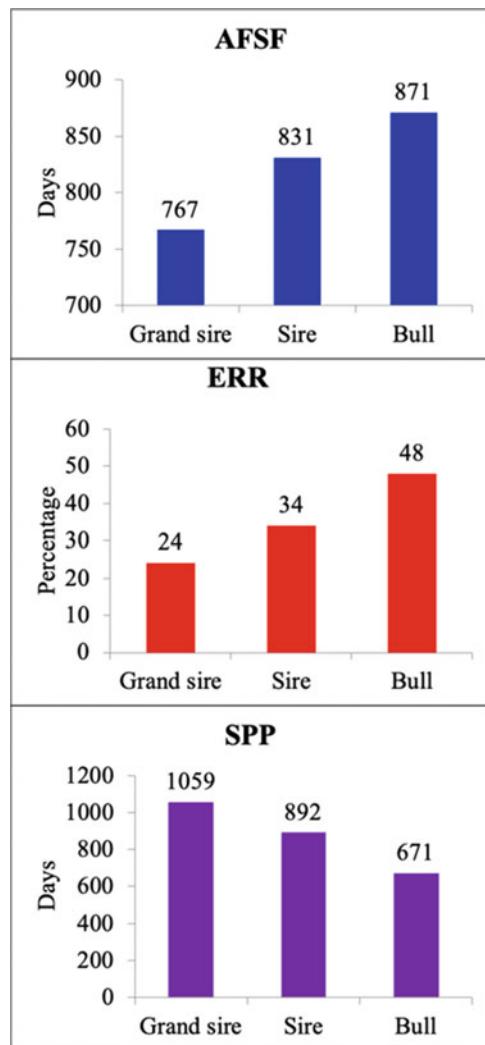
increased feed consumption (due to restrictions in feed intake and appetite). Therefore, the remainder requirements are being met by mobilization of body reserves resulting in negative energy balance (NEBAL). The NEBAL makes the animal conducive to metabolic disorders, which further leads to immune compromise condition and uncoupling of somatotropic axis. High producing cows in low body condition score (BCS) at the time of calving, or that suffer excess loss of BCS during early postpartum, are less likely to ovulate and thereby leading to an impaired reproduction.

- *Delay in resuming cyclicity*: Low BCS coupled with severe negative energy balance affects the pulsatile secretion of luteinizing hormone (LH), reduces the

Fig. 2.2 Possible ways by which higher milk production could be associated with impaired reproduction in bovines
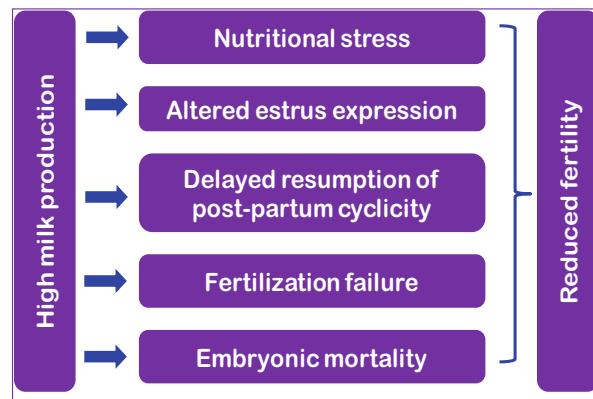
responsiveness of ovaries to LH stimulation, reduces the follicle functional competence, reduces the estradiol production, and ultimately leads to abnormalities in ovulation.

- *Altered estrus expression*: Normal estrous cycles, estrus duration, and overt signs of estrus are essential for deciding the appropriate time of insemination in relation to ovulation so that conception occurs. However, high producing dairy cows have been reported to have shorter estrus (6.2 h Vs 10.9 h), reduced total standing time (21.7 s Vs 28.2 s), and lower peripheral estradiol concentrations (6.8 pg/ml vs 8.6 pg/ml) as compared to low producing dairy cows (Lopez et al. 2004).
- *Fertilization failure*: High producing cows are highly sensitive to heat stress, which contributes to the fertilization failure in these animals.
- *Embryonic mortality:* It is reported that oocytes derived from high genetic merit cows for milk production showed inferior capacity for in vitro embryo development as compared to the oocytes derived from cows of medium-genetic merit, irrespective of the actual milk production (Snijders et al. 2000). Moreover, in lactating high producing cows, there may be asynchrony in the transport of embryo to the uterus that may result in early embryo mortality.

2.5 What Could Be the Approach to Improve Fertility in Dairy Animals?

Having understood the negative impact of high milk production on several physiological pathways, thereby reducing the likelihood of establishment of pregnancy in high yielding cows, the question in front of us is what interventions are needed to improve the fertility in high yielding dairy animals. The interventions may possibly be classified into short- and long-term interventions.

- *Long-term intervention:* Since selection for milk production with little consideration for reproduction traits in the modern dairy cattle has resulted in an antagonistic relationship between milk yield and reproductive performance, due

consideration should be given to traits associated with reproduction while selecting the animals for high milk production.

- *Short-term intervention:* Fertility in dairy animals can be improved using potential reproductive technologies like semen from high fertility sires, controlled breeding, postinsemination fertility enhancement treatments, and assisted reproductive technologies.

2.6 Reproductive Biotechnologies: Development and Applications

Generally, the reproductive biotechnologies are classified into different generations based on the time of development and application (Fig. 2.3). The *first-generation* reproduction biotechnology that has played an unequivocal role in genetic improvement and production enhancement, at least in large animals, is *artificial insemination* (AI) with cryopreserved semen. This technology was born out of the research carried out before 1960s and the progress of this technology has been greatest in several continents. This technology played an important role in animal breeding, especially in the bovines, and brought a visible impact in several countries. The *second-generation* reproduction biotechnology, that is, *multiple ovulation and embryo transfer* (MOET), is more recent and has been in use for several years. In developing countries, the use of this technology is limited, restricted to few farms and organizations that use this technology for production of elite males and females. The cost of the initial investment in establishing facilities and recurring costs for this technology is higher than that of AI, which explains, at least in part, for its less frequent use. In other parts of the world, this technology is included in genetic

Fig. 2.3 Developments in reproductive biotechnologies; the technologies are classified based on their time of development into different generations
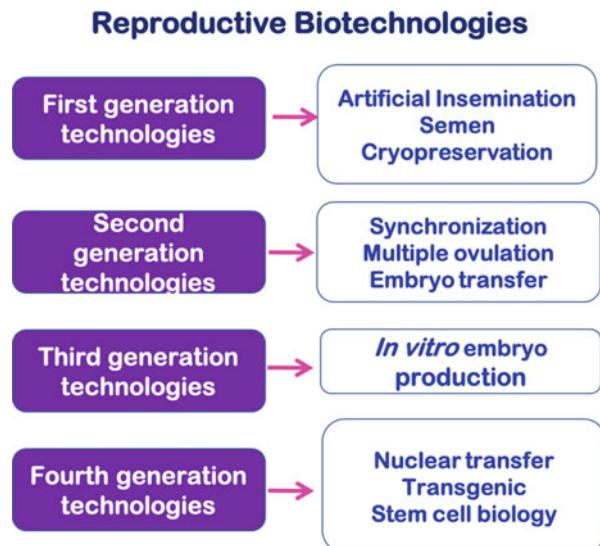
improvement projects and the majority of AI bulls in the world are born out of this technology. The *third generation* reproduction biotechnology, that is, *in vitro fertilization*, is more recent and in use from late 1980s. This technology involves collecting the oocytes very easily at the slaughterhouse as well as collecting the oocytes from live animals through "ovum pick-up." High investments and problems associated with embryo freezing have been the reason for limited use of this technology. However, in considerable number of countries, this technique has really expanded; for instance, Japan and Brazil alone account for about one-half of all the in vitro fertilized and transferred embryos in the world. The *fourth generation* reproduction biotechnology, *nuclear transfer and transgenesis*, is the latest development and mostly restricted to laboratories. The first animal produced by somatic cell cloning was a sheep—"Dolly" and since then – cloning has been carried out in various animals such as cattle, pig, goat, horse, buffalo, and camel.

Other techniques associated with these technologies have immense role in success of the purpose of using these technologies, which include sperm sexing, biomarkers for fertility prediction, gamete cryopreservation, quality improvement of frozen spermatozoa, and fertility improvement protocols. Because the heritability of reproductive parameters is low, application of reproductive biotechniques appears to be an option, which deserves further exploration to inverse the current trend of decreasing fertility in dairy cattle.

2.7 Potential Reproductive Technologies Currently Used in Large Scale

Timed artificial insemination programs: Maximizing estrus detection accurately and efficiently can improve overall reproductive efficiency in bovines. However, at times, estrus detection by visual observation becomes difficult, because a majority of the animals show the peak estrus activity during night. This leads to difficulties in determination of the actual time of onset of standing estrus, and therefore, the possibilities of performing improperly timed AI are high. Therefore, various estrus induction, estrus synchronization, and timed insemination (TAI) protocols have been developed to improve the conception rate. Also, estrus induction protocols can be applied on postpartum anestrus to bring them into cyclicity so that they can be bred during the breeding period resulting in shorter calving to conception intervals. Generally, prostaglandin F2α is used to reduce the lifespan of the corpus luteum and to bring the animal into heat. Regularly cycling animals have a functional corpus luteum during the luteal phase of the estrus cycle. Administration of PGF2α from 5 to 15 days of estrous cycle regresses the active corpus luteum and the animal enters into the follicular phase. PGF2α is not effective when administered on 0–4 days of the estrous cycle. Generally, the treated animals exhibit estrus within 2–5 days of treatment. The protocols for estrus induction using PGF2α and timed insemination are given in Table 2.3.

In the recent past, several protocols have been developed and/or modified to allow TAI so as to circumvent the practical difficulties associated with estrus detection.

Table 2.3 Estrus induction and timed AI using prostaglandin $F_2\alpha$

Day of treatment	Two injection method	One injection method without palpation	One injection method with palpation	
Day 0	$PGF_2\alpha$ injection	$PGF_2\alpha$ injection	Rectal examination for presence of CL	
			CL present—$PGF_2\alpha$ injection	CL absent—No treatment
Day 3 & 4	--	Breed the animals in heat	Breed the animals in heat	–
Day 11	$PGF_2\alpha$ injection	$PGF_2\alpha$ injection in animals not inseminated	–	CL present—$PGF_2\alpha$ injection
Day 14 and 15	Insemination at 14th day and /or 15th day (72 and 96 h after $PGF_2\alpha$ injection)			

Some of the protocols have advantage of synchronizing ovulation as well and they can be applied on large scale to improve the fertility in dairy animals. Different protocols are given in Fig. 2.4. Ovsynch protocol is one such, in which gonadotropin-releasing hormone (GnRH) is administered on day 0 of start of treatment (irrespective of the stage of estrous cycle), prostaglandin F2α is administered on day 7, and again GnRH is administered on day 9. The animal may be inseminated at 16 to 22 h after the administration of second GnRH. The first GnRH injection induces ovulation of the ovarian follicle and subsequent development of the corpus luteum. Depending upon the state of maturation of follicles at the time of GnRH administration, the rate of ovulation induced by the first GnRH injection varies between 65% and 85% (Pursley et al. 1997). The success rate of this protocol is high and being practiced regularly at several farms. A modification of Ovsynch protocol, called as Doublesynch protocol, has been developed by incorporating an additional $PGF_2\alpha$ administration at 48 hour before the start of Ovsynch protocol and claimed to yield superior results. The Controlled Internal Drug Release (CIDR) – GnRH protocol – involves CIDR insertion on day 0 and removal on day 7. GnRH is administered on day of CIDR insertion. On the day of CIDR withdrawal, PGF2α is administered and after 2 days of PGF2α administration, the second dose of GnRH is administered. The advantage of inclusion of CIDR in GnRH-based programs is that the animal is under the influence of progesterone during the period between day 0 and day 7, which will prevent early onset of estrus and ovulation. Although all these protocols have been shown to improve fertility, it is important to remember that nutritional status of the animal, sanitary management, and skill of the operator can influence the success rates significantly. Further, it should be remembered that if these protocols are used for all cows without gynecological examinations, some animals may not respond due to undetected reproductive disorders such as true anestrus, ovarian cysts, and subclinical endometritis (Nowicki et al. 2017).

Multiple ovulation and Embryo Transfer: In several countries, embryo transfer (ET) technology has played a vital role in genetic improvement of dairy cattle over
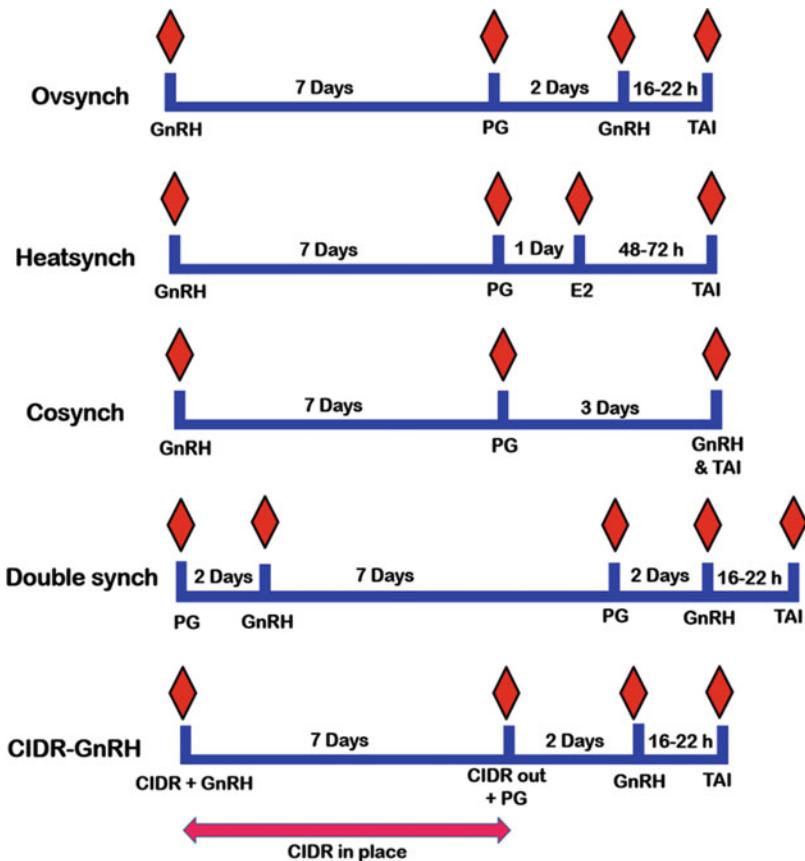
Fig. 2.4 Different protocols used for timed AI and synchronization of ovulation with insemination

the past several decades. The advantage of this technology in terms of genetic improvement is not only through the production of cows, but also through the production of bulls for utilization in the artificial breeding program. In USA, 99% and 95% of currently available Holstein and Jersey AI sires, respectively, were produced using ET (Sommer and Youngs 2016). Developments in superovulation protocols and nonsurgical embryo transfer have made MOET technology viable for commercial application. Across the globe, as per the data of International Embryo Transfer Society, a total of 1.41 million cattle embryos (both in vivo derived and in vitro produced) were produced in the year 2019. North America alone accounted for around 52% of the total embryos produced. In recent years, the total number of in vitro produced embryos surpassed the total number of in vivo derived embryos. The increase in the proportion of in vitro produced embryos actually transferred suggests progress in in vitro embryo production systems adopted worldwide. Although such data are not available in several developing countries, existing sporadic reports indicate limited use of MOET in these countries. For instance, in

Fig. 2.5 Higher number of transferable embryos obtained from a 14-year-old dual purpose cow (Deoni breed). Upper—the donor cow; Bottom—embryos

India, National Dairy Development Board established ET facility in 1987 and ET technology has been used in bull production programs. Similarly, several research and development institutes also started MOET, although not in large scale, and have shown considerable progress. However, the technology needs to be standardized to specific breeds and then implemented at large scale. For instance, it is believed those older cows (>10 years) are generally not to be used for superovulation as the expected embryo recovery is low. However, a recent report from the Southern Regional Station of ICAR-NDRI, Bengaluru, India, shows that MOET was carried out in Deoni breed (a dual-purpose breed of cattle) and obtained 14 transferable embryos (Fig. 2.5) from an old Deoni cow (13.6 years age), indicating that indigenous breeds might have higher oocyte reserves.

In vitro *embryo production and transfer:* An alternate method to superovulation and embryo collection from donor cows is in vitro embryo production. This method involves the steps like collection of the oocytes, in vitro maturation, in vitro fertilization, and in vitro culture of embryos till they reach the stage that they can be

transferred to the recipient animals. Oocytes can be collected either from the ovaries obtained from slaughtered animals or from live animals. Ovum Pick-up (OPU) from live animals and in vitro embryo production gained momentum during recent years. OPU is a nonsurgical technique in which oocytes are aspirated from the ovaries of live animals using ultrasound and a guided needle. Combined application of OPU and in vitro embryo production is one of the recent breakthroughs in animal reproduction for improving reproductive efficiency and for quick propagation of elite germplasm. The cow's ovary contains several thousands of oocytes, out of which she may ovulate only about 200 oocytes in her lifetime. Normally, after every estrus, a cow ovulates only one oocyte, but at any given point of time, during estrous cycle, up to 50 antral follicles exist on the ovary. On a conservative estimate, using OPU, 15–20 oocytes can be collected per week from a valuable donor cow, which translates into potential collection of about 700–1000 oocytes/year/cow. Out of these oocytes, 200–300 blastocysts or 80–120 pregnancies can be obtained in a year with the assumption that the blastocyst rate is 30% and a pregnancy rate of 40%. However, to exploit its full potential, a reliable in vitro fertilization system and a dedicated OPU team are needed. As per the data of International Embryo Transfer Society, a total of more than one million embryos were produced using Ovum Pick-up and in vitro embryo production in the year 2019.

Use of sexed semen: Normally ejaculates from a bull contains both X chromosome bearing (when take part in fertilization, results in birth of female offspring) and Y chromosome bearing (when take part in fertilization, results in birth of female offspring) spermatozoa. Thus, insemination of a cow with traditional frozen semen straws may result in either female or male calf. However, owing to the developments in techniques and technologies, now, it is possible to separate (sort) X chromosome bearing spermatozoa from Y chromosome bearing spermatozoa. Thus, insemination of a cow with X chromosome bearing spermatozoa–enriched semen is expected to result in birth of female calf. As such, no technology is now available to sort X chromosome bearing spermatozoa from Y chromosome bearing spermatozoa with 100 percent efficiency. Fluorescence-activated cell sorting, a specialized type of flow cytometry, is the only commercial method of sperm sex sorting available as on date and the success rate (in producing desired sex of calf) with this technology is around 90–95 percent. However, several reports indicate that the conception rates with sexed semen are lower than the conception rates with unsexed semen. Efforts are ongoing to minimize the sperm damages associated with the sorting procedure and to enhance the conception rates with sexed semen. By using the sexed semen enriched with X chromosome bearing spermatozoa for artificial insemination (AI), it is possible to substantially increase the number of elite females in short time period. Therefore, artificial insemination using sexed semen is a pragmatic and easy way for preselection of the sex of the offspring. Selective use of sexed semen will increase the genetic progress from the daughter-dam path and help in producing superior males from elite bulls for future breeding. Combining MOET/IVEP with sexed semen is further advantageous to multiply superior germ plasm in a shorter time.

2.8   Perspective and Prospective

The ultimate success of the dairy industry depends upon how economically the dairy animals are produced and managed, which in turn is directly influenced by the herd reproductive efficiency. Poor dairy bovine fertility continues to remain as one of the most significant challenges faced by the majority of dairy farmers across the globe. Over the past few decades, significant changes have occurred in dairy bovine production in terms of greater herd size per farm and intense selection of animals for milk production that resulted in increased milk production. To manage high producing bovines, the nutrition and management have changed considerably over the last few decades, but due consideration was not given to the long-term consequences of these nutritional and managemental changes on the basic reproductive physiology of the animal. As a consequence, several studies across the globe have reported a decrease in the fertility of dairy bovine. However, on the other hand, some of the highest yielding and most productive herds in some countries are able to maintain optimum fertility. This clearly indicates that poor fertility is not an inevitable consequence of high milk production in dairy cattle.

Although reproductive parameters have low heritability, by applying careful selection strategy, it is possible to maintain high production as well as reproduction efficiency in dairy animals. Genomic selection combined with advanced reproductive technologies has accelerated the rate of genetic progress in dairy cows. However, the inherent difficulties in selecting animals for high fertility include (i) routinely measured reproductive traits are not very accurate (ii) reproductive traits have low heritability, (ii) animals genetically superior for production tend to have inferior reproduction, and (iv) reproduction traits are polygenic and effect of one gene may depend on others. Mostly days open, cow and heifer conception rate are the three female fertility traits used in genetic evaluations of dairy cattle. Recently, SNPs associated with daughter pregnancy rate, first service pregnancy rate, services per conception, and days open have been identified. However, further research is required to estimate specific parameters for fertility traits and determine whether selection for individual components can affect more rapid genetic change in fertility than direct selection for days open. Recent research identified potential markers for ovarian reserve, cyclicity, conception, fetal growth, calving ease, and postpartum reproductive efficiency. Application of such markers could be valuable in selection of dairy animals for high fertility. On the other hand, the application of reproductive biotechniques within the framework of an adapted reproductive health program could be a viable option to reverse the trend of decreasing fertility in dairy bovines.

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Role of Kisspeptin in Bovine Reproduction: Concepts and Applications

3

Mohan Mondal, Adamou Akourki, and James J. Ireland

Abstract

Kisspeptin, a product of KISS1 gene, is a RF-amide neuropeptide with high affinity to KISS1R (receptor of KISS1 gene), previously called as orphan G protein–coupled receptor (GPR54). Kisspeptin was initially reported to be expressed in nonmetastatic melanoma cells with a subsequently powerful suppressor role of the metastatic melanoma. Since its discovery in 1996, kisspeptin is regarded as a potential regulator of hypothalamo-pituitary-gonadal (HPG) axis with expression in many organs and tissues. Therefore, KISS1-KISSR system is reported as one of the important gatekeepers for numerous physiological processes like puberty, various reproductive and metabolic functions, and also some pathophysiology conditions. This book chapter provides an overview of our current knowledge on kisspeptin-kisspeptin receptor system with especial focus on the role of kisspeptin-KiSS1/R system in bovine reproduction.

Keywords

Kisspeptin · Bovine · Hypothalamus · Reproduction regulation · Puberty

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3.1 Introduction

It is well understood since long time that onset of puberty and subsequent reproductive processes are the consequences of interactions of the hypothalamo-pituitary-gonadal (HPG) axis mainly through the stimulation of gonadotrophin-releasing hormone (GnRH). The GnRH is synthesized in the preoptic area of the hypothalamus and then released into the median eminence from axon terminals in a pulsatile manner to stimulate the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the adenohypophysis, which ultimately act on the gonads to boost gametogenesis and synthesis of sex steroids. The GnRH stimulation is provoked by a number of important activating factors that could interact together or partially like phoenixin (Yuan et al. 2017), kisspeptin (Hendriks et al. 2017), neurokinin B (Navarro et al. 2015), leptin (Ohga et al. 2015), etc. Out of these GnRH stimulators, kisspeptin is considered to be a most potent GnRH-secretagogue in mammalian species. The regulation of HPG axis by kisspeptin was known later in 2003 than its first known function discovered by Lee et al. (1996) who found that transfecting a full-length KiSS-1 cDNA into metastatic cells could clearly suppress metastasis. Since then, researchers are exploring the influence of kisspeptin on various physiological and pathological events emphasizing its role on reproduction. Therefore, some of these highlight the most recent updates addressing the metastatic role of KISS1 that was expressed in nonmetastatic melanoma cells and was subsequently demonstrated to act as a powerful suppressor of the metastatic potential of malignant melanoma cells in different types of cancer, including breast, pancreatic, ovarian, gastric, prostate and bladder cancer, and esophageal carcinoma (Bhattacharya and Babwah 2015; Wahab et al. 2016). Others emphasized the current advancement on molecular diversity and phylogenetic evolution of vertebrate traits of kisspeptin, KiSS1 and KiSS2, and their respective receptor genes, that is, GPR54–1 and GPR54–2 (Kanda and Oka 2013; Yun et al. 2014). Furthermore, another group of scientists had focused on the electrical properties of kisspeptin neurons and neurohormonal regulation of kisspeptin activity by its topographic distribution, network connectivity, neurochemistry, and burst firing of kisspeptin and kisspeptin-R system (Shannon and Alexander 2017). Meanwhile, the oscillation of kisspeptin neurons on a circadian basis and its expression clock genes that are thought to regulate its rhythmic activities were also aimed by many researchers (Beymer et al. 2016).

Recently, most of the researches on kisspeptin have been associated to delineate its roles in reproduction, where kisspeptin-receptor signaling has been described in various physiological and pathophysiological conditions like during the onset of puberty and in the genital tract, and more for its possible therapeutic application in the treatment of fertility disorders (Okamura et al. 2013; Clarke et al. 2015; Clarke and Dhillo 2016; Nejad et al. 2017). Other attributed functions to kisspeptin, no less important, were metabolism and stress regulation of kisspeptin in the modulation of reproductive function and sexual, social, and emotional brain processing integrated by kisspeptin (Alexander and Dhillo 2017; Comninos et al. 2017).

Among all multifaceted roles of kisspeptin as mentioned above, this chapter will emphasize specifically the role of kisspeptin in reproduction of livestock species in general and bovines in particular. Finally, we will throw some light on how kisspeptin, a potent secretagogue of GnRH, can be used to address fertility issues of livestock species in future.

3.2   Discovery and Structure of Kisspeptin

Initially, kisspeptin was been considered as a biomolecule (known then as metastin) responsible for the suppression of metastasis. This important invention was made in 1996 (Lee et al. 1996) using a "modified subtractive hybridization" method for the purpose of comparing the mRNA expressions through analyzing the cDNAs in metastatic (C8161/MelJuSo) as well as nonmetastatic cells/hybrid clones (neo6/C8161 or neo6/MelJuSo) in chromosome 6/melanoma cell hybrids. The study revealed a suppression of metastasis in an expression-dependent manner when the full-length KiSS-1 cDNA was transfected into C8161 melanoma cells. One year later, the authors confirmed that the KiSS-1, when injected (as transfectant of KiSS-1 clones) into the mammary fat pads of athymic nude mice, acts as a metastasis suppressor gene in some of the human breast cancers tested (Lee and Welch 1997). The KiSS-1 cDNA was reported to encode a 164 amino acid hydrophilic peptide enriched with a polyproline domain containing an SH3 ligand and a putative protein kinase C-alpha phosphorylation site (Lee et al. 1996). Later, a corrected genomic sequence and structure, and refined chromosomal location for KiSS-1, were proposed (West et al. 1998). They reported four exons for this KiSS-1 gene. Of which, the first two are not found to be translated; the third one was reported to have 38 5′ noncoding bases that follow the translational start site with another 100 translated bases. The last exon, that is, terminal one, contains 332 translated bases, the translational stop codon, and the polyadenylation signal. Afterward, other studies were performed showing KiSS-1 with high-affinity natural agonists of GPR54 (Kotani et al. 2001) and encoding a carboxy-terminally amidated peptide with 54 amino-acid, primarily isolated from human placenta as the endogenous ligand of GPR54 named as "metastin" (Ohtaki et al. 2001). The GPR54 was identified in the rat brain as an orphan receptor exhibiting high sequence similarity of 45%, 45%, and 44%, respectively, with the transmembrane regions of galanin receptors GalR1, GalR3 and GalR2 (Lee et al. 1999). Furthermore, other kisspeptin fragments that were identified from its precursor were named based on the number of amino acids they contain like kisspeptin-10, kisspeptin-13, and kisspeptin-14 (Bilban et al. 2004; Fig. 3.1). And, it was found that all the fragments of kisspeptin staring from kisspeptin-54 to kisspeptin-10 (the shortest kisspeptin fragment) have similar affinity and efficacy on its receptor, indicating that the C-terminal part of the peptides is responsible both for the high-affinity binding and the activation of GPR54 (Kotani et al. 2001). Unlike humans and animals, the identification and characterization of the fish gonadal kisspeptin system revealed four kisspeptin system mRNAs, that is, kiss1, kiss2, and gpr54–1 and gpr54–2 (Fairgrieve et al. 2016).
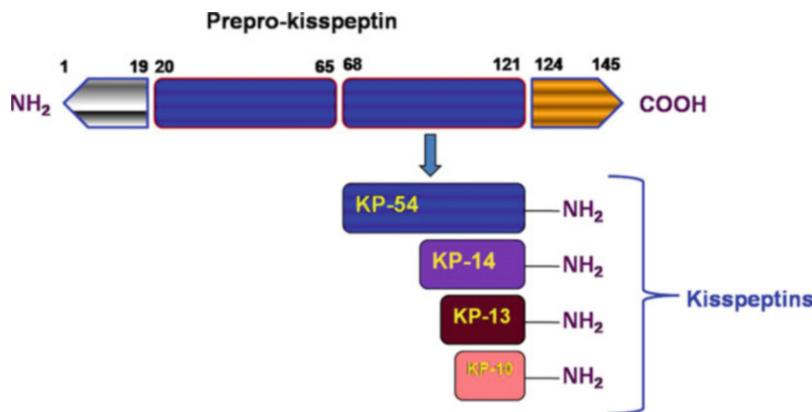
Fig. 3.1 Formation of different types of kisspeptin from its common precursor. Structurally different types of kisspeptin are produced by the cleavage from its common precursor, that is, the prepro-kisspeptin. The prepro-kisspeptin, a peptide of 145 amino acid long, consists of a signal peptide (19-amino acid peptide) and a central 54-amino acid region, that is, kisspeptin-54 (previously known as metastin). Further cleavage of kisspeptin-54 produces kisspeptins of lower molecular weight: Kp-14, Kp-13, and Kp-10. All kisspeptins contain the RF-amide motif that is required to bind and activate KiSS1R [Adopted and modified from Hu et al. 2018]

3.3 Localization of Kiss1/Kiss1r

Kisspeptin neurons are generally located in the hypothalamus, but kisspeptin receptors are localized in different parts of the brain, pituitary, genital tract, and placenta of the vertebrate animals and this localization depends on the species and physiological stage of the animals.

3.3.1 Hypothalamus

Kisspetin was found in the arcuate nucleus (ARC) and medial preoptic area (mPOA) in sheep where the bed nucleus of the stria terminalis (BNST) was responsible for sexual behavioral onset, whereas the ARC, ventromedial nucleus (VMN), mPOA, and kisspeptin cells located in the ARC and mPOA were found to regulate the positive feedback of estradiol only during the LH surge (Franceschini and Desroziers 2013). Similar distribution map of kissppetin10-immunoreactive (Kp10-ir) cells in the ovine hypothalamus was reported (Franceschini et al. 2006). Indeed, high numbers of KP10-ir cells were present in the caudal ARC, mPOA, and dorsomedial nucleus, and numerous varicose KP10-ir fibers were found in the preoptic area where GnRH neurons reside and in the median eminence, seemingly projecting around small capillaries in its external zone. Expression of KiSS1 in the ARC across the ovine estrous cycle was found to increase in the caudal ARC during the preovulatory period (Estrada et al. 2006). The chronic levels of estrogen and progesterone were

found to negatively regulate the expression of the KiSS1 gene in the ARC of the ewe, suggesting that both these steroids may influence GnRH secretion by exerting negative feedback control via altered kisspeptin signaling (Smith et al. 2007). Polkowska et al. (2015) reported a decrease of kisspeptin axons extending from preoptic area to medial basal hypothalamus and also in the median eminence in the lambs that were fasted for short term compared to control animals. Recently, it was demonstrated that the density and proportion of these kisspeptin neurons in ARC and POA were increased with the ram effect in sexually quiescent ewes (Fabre-Nys et al. 2017).

3.3.2 Pituitary

Kisspeptin gene is expressed in different cells of the pituitary. KiSS1 and KiSS1R are expressed throughout the gonadal development (Shahi et al. 2016). The most recent advances of the role of the Kiss1/Kiss1R system regulating pituitary functions emphasizing the direct role of kisspeptin on pituitary cells and its interactions with other relevant regulators have been reviewed recently (Gahete et al. 2016).

3.3.3 Genital Tract

Kisspeptin/KISS1R is largely expressed in genital tract of male and female (Fairgrieve et al. 2016; Shahi et al. 2016; Rather et al. 2017).

The very first study on KP-KiSS1-KiSS1R system revealed that KiSS-1 and KiSS-1R mRNA expressed more abundantly in the placenta (Lee et al. 1996; Muir et al. 2001; Bilban et al. 2004). Kisspeptin-ir was also found in the cyclic ovaries, where prominent signals were recorded in the theca layer of growing follicles, corpora lutea, interstitial gland, and ovarian surface epithelium (Gaytán et al. 2009). KISS1 and KISS1R were also expressed in the uterus, ovary, and oviduct with marked expression in endometrial and oviductal epithelial cells throughout the reproductive cycle (Roman et al. 2012). In rodent female, it was found that the WT mouse uterus expresses kisspeptin and KISS1 receptor on day 4 of implantation (Babwah 2015). There are also evidences, which clearly showed that kisspeptin signaling is essential for folliculogenesis, oocyte maturation, and ovulation (Gaytan et al. 2014; Hsu et al. 2014).

Studies conducted in crossbred cows showed for the first time a large and interesting data not only on development of a simple and sensitive assay for kisspeptin determination in plasma but also on kisspeptin concentrations during follicular development and pregnancy (Mondal et al. 2016a). Indeed, these authors recorded three peaks of kisspeptin during an entire estrous cycle; the first peak was found to occur on a day before appearance of preovulatory LH surge; the second and third peaks were found on day 6 and 18 of the estrous cycle (Fig. 3.2). This kisspeptin level increased with the follicular development and with the stages of pregnancy. Another study conducted in mithun (*Bos frontalis*) showed that
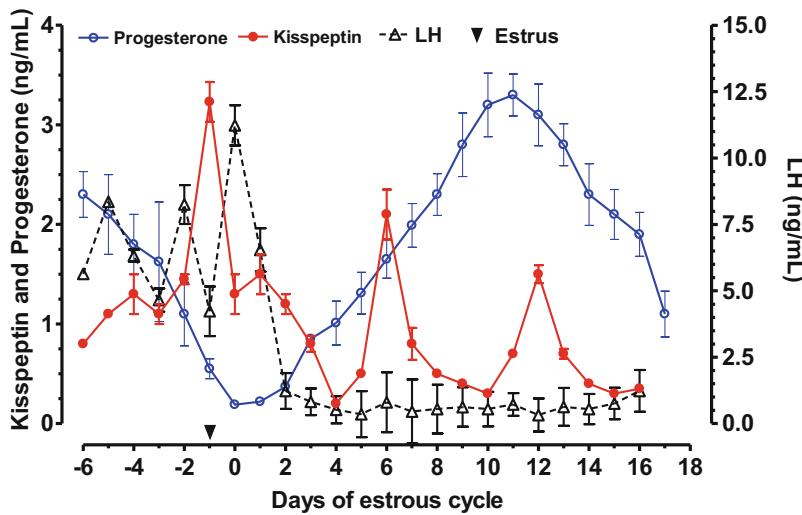
Fig. 3.2 Blood kisspeptin, LH, and progesterone profiles (mean ± SEM) in cyclic crossbred cows. On an average, a total of three peaks of kisspeptin were recorded during an entire estrous cycle; the first peak was found to occur on a day before appearance of preovulatory LH surge, the second and third peaks were found on day 6 and 18 of the estrous cycle. Kisspeptin peaks in an estrous cycle vary from 2 to 4. [Adopted from Mondal et al. 2015]

kisspeptin concentrations in follicular fluid increased as the follicles grow and the highest concentrations were found in preovulatory follicles (Mondal et al. 2016b).

In mammalian male, KISS1/KISS1R system plays an important role in the physiology of the genital organ/tract development. KiSS1 and its receptor are expressed mainly in Leydig cells and seminiferous tubules, respectively (Hsu et al. 2014). Moreover, the expression of KISS1R was later observed in the gamete, specifically in the acrosome of spermatids and mature sperm. Reduction of serum kisspeptin levels was reported after gonadectomy in males (Salehi et al. 2015). Also, Kisspeptin and its receptor were detected in the interstitial compartment and Sertoli cells in the periphery of the seminiferous tubules in adult testes (Irfan et al. 2016).

Kisspeptin receptor, besides its high presence in placenta and pituitary, was also expressed in pancreas and spinal cord, which clearly indicates its role in the control of the endocrine function (Kotani et al. 2001). For the nonvertebrate species, existence and distribution of kisspeptin-like peptides (Kiss-l) and their receptors were discovered in subesophageal, thoracic ganglia, dorsolateral cluster, and ventromedial cluster in the central nervous system (Thongbuakaew et al. 2016).
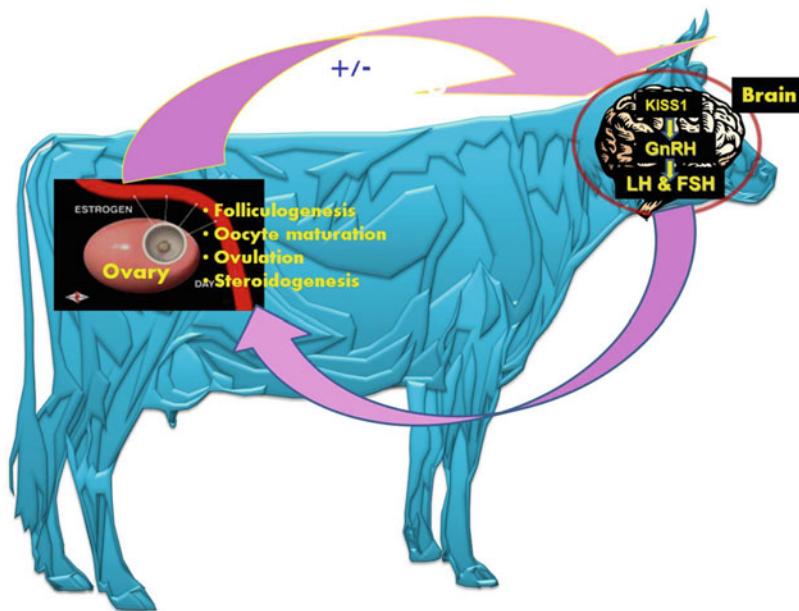
Fig. 3.3 Influence of kisspeptin on ovarian functions and its control through steroid feedback mechanism in bovine species. The main functions of kisspeptin in domestic animals on ovary are folliculogenesis, oocytes maturation, ovulation, and steroidogenesis. Kisspeptin neurons are found to be controlled by the levels of estrogen. Low level acts as inhibitory and high level acts as stimulatory of the kisspeptin neurons. Progesterone always exerts inhibitory action like that of low level of estrogen

3.4 Regulation of Kisspeptin by Sex Steroids

Research findings showed that kisspeptin neurons are regulated by sex steroids (Fig. 3.3). Experiments conducted in ovine species clearly showed that low and high levels of estrogen regulate the kisspeptin neurons in an inhibitory and stimulatory manner, respectively. Progesterone and testosterone was also found to inhibit the kisspeptin neurons as that of low level of estrogen. The concentration of estrogen required to exert a negative feedback on GnRH secretion is also capable to inhibit kisspeptin neurons in the ARC (Lopez et al. 2016). Abundance of transcripts encoding KISS1 gene was found to increase in the ARC during the late follicular phase of the estrous cycle than other phases (Estrada et al. 2006; Li et al. 2015). Treatment of OVX ewes with exogenous estrogens to induce a surge of LH resulted in an increase in Fos expression in arcuate kisspeptin neurons, thereby leading to neuronal activation (Smith et al. 2009). The activation of kisspeptin neurons was reported to be much higher in estrous animals than the ewes in the luteal phase (Merkley et al. 2015). Hence, unlike female rodents, where the arcuate kisspeptin population is negatively regulated by estrogen (Smith et al. 2005), both positive and

negative regulations of the arcuate population exist in the ruminants like ewes depending on the circumstances.

Unlike estrogen, less report is available on the regulation of kisspeptin by progesterone. Ovariectomized ewes administered with progesterone showed moderately low level of KISS1 mRNA than to non-OVX animals, which had very insignificant effect than those treated with estrogen (Smith et al. 2007). Very limited studies have been conducted in large ruminants like cattle to compare the population of kisspeptin neuron during follicular and luteal phases of the estrous cycle. Studies revealed that population of kisspeptin cell in the preoptic (Hassaneen et al. 2016) and arcuate area (Tanco et al. 2016) was higher during the follicular than luteal phase of the bovine estrous cycle. These studies suggested that progesterone generally inhibits kisspeptin expression in the arcuate nucleus.

3.5 Kisspeptin and Reproduction

3.5.1 Puberty

"Puberty is major developmental event in the lifespan of any individual, which culminates with the attainment of sexual (somatic, psychological) maturity and reproductive capacity. This intricate maturational phenomenon is grounded on early differentiation events (starting *in utero*), and involves a complex series of morphological, functional, endocrine, behavioral and psychological changes, which ultimately lead to the acquisition of a complete adult phenotype" (Parent et al. 2003). Physiologically, puberty in animals could be considered as "the developmental processes, which begins with the first sign of ovarian activity that terminates with the onset of the first reproductive cycle" (Blaustein et al. 2016). This growth phase in the vertebrates begins with the increased pulsatile secretion of GnRH that ultimately results in gonadal function activation required for the onset of puberty (Teles et al. 2011). While the multifactorial control of GnRH neurons is well established, and GnRH secretion has been reported to be regulated by several neuropeptides and transmitters, kisspeptin has recently been considered as a potential regulatory signal to control the onset of puberty in mammalian species (Pinilla et al. 2012). It has been seen that for final activation of GnRH neurons during pubertal process, higher expression of hypothalamic KiSS1 mRNA and kisspeptin peptide is utmost essential. Enhanced kisspeptin pulsatility with higher frequency and amplitude has been recorded during pubertal onset in crossbred cows (Mondal et al. 2021—*unpublished data*). Repeated administration of kisspeptin-10 in prepubertal crossbred heifers hastens puberty at least 2 months earlier than control animals (Mondal et al. 2021—*unpublished data*). Similarly, early activation of the gonadotrphic axis and induction of precocious vaginal opening were possible in immature female rats treated with repeated doses of exogenous kisspeptin-10 (Navarro et al. 2004b) and showed an increased Kiss-1 mRNA coinciding with the pubertal onset (Navarro et al. 2004a). The expression of hypothalamic Kiss-1 mRNA reached the highest level on the day when the animal attained puberty. On the other hand, the population

of kisspeptin-ir cell in both ARC and PeN was found to decrease, but the population of the cell was increased in POA during the postpubertal period (Sun et al. 2007). And also, it was seen increasing the number of kisspeptin cells in ARC nucleus, periventricular nucleus and POA, and Kiss1 mRNA in the hypothalamus as soon as puberty is attained (Takase et al. 2009; Cui et al. 2015). This action of kisspeptin on puberty is possible by modulating the activity of estrogen circuits at the onset of puberty (Cui et al. 2015). Expression of KiSS1 and KiSS1R has been increased during the transition period from prepubertal to pubertal stage in crossbred cows and sheep (Redmond et al. 2011; Mondal et al. 2021—*unpublished data*). KiSS1 mRNA levels were also found to increase across the early stages of puberty in the preoptic area in OVX ewe lambs treated with estrogen and a high positive correlation that was found to exist between the number of KISS1-expressing cells and the frequency of LH clearly indicates their active involvement with the process of onset of puberty (Redmond et al. 2011).

3.5.2 Regulation of Gonadotropins, LH Surge, and Ovulation by Kisspeptin

Most of the research in the field of kisspeptin biology of domestic animals has been concentrated on the effects of exogenous kisspeptin administration on its efficacy to release plasma LH. Single intravenous kisspeptin administration has been found to increase blood LH in females from all livestock species studied so far like cattle (Kadokawa et al. 2008), goats (Hashizume et al. 2010), sheep (Caraty et al. 2007), horses (Magee et al. 2009), and pigs (Lents et al. 2008). Kisspeptin probably acts at three places to stimulate plasma LH: (a) stimulating GnRH secretion through acting on the GnRH neurons present in the POA or ARC nucleus, (b) stimulating on GnRH terminals present in the median eminence, and (c) stimulating LH secretion by directly acting on adenohypophysis (anterior pituitary). And, all these can act as GnRH/LH pulse generator and also have influence on the preovulatory GnRH/LH surge.

A number of studies revealed that exogenous administration of kisspeptin always resulted in increased release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the blood. In goats, it was seen that the average concentrations of plasma LH and FSH in the treatment group were significantly higher 6 h after kisspeptin analog treatment than those in the control group. And also, for the second ovulation, ovulatory follicles in the treatment group were significantly smaller in maximal diameter than in the control group (3.8 ± 0.5 vs 5.4 ± 0.2 mm, $p < 0.05$) (Goto et al. 2014). The timing of surge-like release of these hormones after kisspeptin injection is associated with the estrous cycle (Endo et al. 2015; Rahayu et al. 2017). Because during the luteal phase, a surge-like release of LH with a peak was observed at 12.5 ± 1.0 h after administration of exogenous kisspeptin, but in the follicular phase, a surge-like release of LH occurred immediately after kisspeptin administration with a peak at 6.0 ± 3.5 h (Endo et al. 2015). Furthermore, significant increases in plasma LH concentration were detected during the periods of 3 to 5 h

and 2 to 5 h after 50 μg kisspeptin analog injection in female goats with early luteal phase and midluteal phase, respectively (Rahayu et al. 2017). But, these increased peripheral concentrations of both LH and FSH in mares were always rapid, transient, and failed to induce ovulation, even when kisspeptin was perfused for prolonged periods (Decourt et al. 2014). Similar observations were made by McGrath and his team 2016, when they found that a 30 hours infusion of kisspeptin at a low and high rate ([88 μg] and [130 μg] per hour, respectively) in mares increased LH secretion but was not able to stimulate an LH surge for follicle ovulation. Also, administration of 500 μg and 1.0 mg rodent kisspeptin (rKP-10) exhibited LH and FSH peak, although a single injection of 1.0 mg rKP-10, i.v., was not enough to induce ovulation in the estrous mare (Magee et al. 2009).

A significant stimulation of LH and FSH release was observed in kisspeptin-treated young calves with peaks of their plasma concentrations reached at 20–40 min after kisspeptin injections (Ahmed et al. 2009). The area under the LH and FSH curve for a 120-min period post-Kp10 administration was significantly higher in the males than females ($P < 0.05$). And this LH and FSH release is greater after an i.v. than an i.m. injection of Kp10 in calves (Ahmed et al. 2009).

3.5.3 Kisspeptin Regulation of Estrous Cycle

Literature on the influence of kisspeptin in estrous cycle of domestic animals is limited. A few studies have been conducted on kisspeptin during rat estrous cycle. Experiments in cattle revealed that kisspeptin concentrations varied significantly ($p < 0.05$) during different days of bovine estrous cycle. A mean of three kisspeptin peaks of varying amplitudes was recorded during the entire bovine estrous cycle. The highest peak of kisspeptin was obtained on 2 days before the onset of estrus (day—2). On an average, the second and third peaks of kisspeptin were found to occur on day 6 and 12, respectively. The number of peaks for blood KP was recorded to vary between two to four in individual cows (Mondal et al. 2015; Fig. 3.2).

Abundance of transcripts encoding KiSS1 gene was found to be significantly different ($P < 0.01$) during different stages of bovine estrous cycle. The highest expression of KiSS1 gene was found during proestrus stage of the estrous cycle. Abundance of transcript encoding KiSS1R gene was similar ($p > 0.05$) during different stages of estrous cycle (Mondal et al. 2021—*unpublished data*).

One study conducted in Japanese Black beef cows showed that exogenous kisspeptin-54 accelerated the follicular development of the first DF and this effect was mediated by kisspeptin-induced LH release (Naniwa et al. 2013). Full-length kisspeptin was also capable of inducing ovulation (Naniwa et al. 2013). Thus, keeping in view the great potential of kisspeptin as a "biomolecule of promise'" for reproduction augmentation, it can be considered as a novel drug of choice to stimulate the follicular growth and development, and ovulation in livestock species in general and bovine in particular.

3.5.4   Kisspeptin and Assisted Reproduction

Kisspeptin controls secretion of GnRH and gonadotropins, thereby controlling the functions of gonads, and this is the basis for using kisspeptin in assisted reproduction in almost all mammalian species including livestock (Macedo et al. 2014, 2015, 2019; Mondal et al. 2015, 2016; Decourt et al. 2016; Northup et al. 2020). Exogenous kisspeptin administration was reported to increase endogenous LH secretion and resulted with better ovulation synchronization than GnRH in crossbred dairy heifers (Mondal et al. 2015) and crossbred beef heifers and cows (Leonardi 2018). Bovine full-length kisspeptin induced LH release and growth of the dominant follicle during first follicular wave in Japanese Black beef cows (Naniwa et al. 2013). Similarly, continuous 48 h administration of kisspeptin through osmotic minipump during proestrus increased growth of the follicles and rate of ovulation in anestrous Nelore cows (Macedo et al. 2015).

Recently, kisspeptin analog, called C6, has pharmacological advantages over endogenous kisspeptin in terms of having a long-lasting effect and increased activity following intramuscular injection (Decourt et al. 2016). The C6 influenced plasma LH and FSH in the Alpine goat during the breeding, nonbreeding season, and at the onset of the breeding season and triggers successful ovulation. The C6 has also been reported to advance puberty in female mice when administered for five consecutive days (Decourt et al. 2016). Hence, C6 or any other kisspeptin analogs can potentially be used for early attainment of puberty and fertility management in livestock species. All the evidences as cited herein clearly revealed that the kisspeptin, either natural or its analog, can stimulate inactive or partially active GnRH neurons, thereby initiating reproductive processes.

KISS1/KISS1R was found to be localized in the follicular cells (granulosa and theca cells), and oocytes, and kisspeptin was also reported to present in the follicular fluid (Mondal et al. 2015; Fig. 3.4). We can, therefore, suggest from these findings that kisspeptin and KiSS1/KiSS1R system may have autocrine/paracrine actions within the follicles. Kispeptin-ir has also been detected in bovine corpus luteum (CL). Young CL was found to contain higher kisspeptin than the mid- or late-cycle CL (Mondal et al. 2015; Fig. 3.5). Supplementation of kisspeptin in the culture media was reported to enhance in vitro maturation of oocytes (Byri et al. 2017) and increased blastocysts formation (Soares et al. 2018) in sheep, cattle, and buffaloes (Rajesh et al. 2018).

3.5.5   Kisspeptin and Pregnancy

In female genital tract, KISS1/KISS1R system plays an important role in regulating implantation of the mammalian embryo, placentation, and maintenance of pregnancy (Lee et al. 1996; Bilban et al. 2004). Moreover, it has been demonstrated that the WT mouse uterus expresses kisspeptin and KISS1 receptor on day 4 of implantation (Babwah 2015). Furthermore, in human, KISS1 and KISS1R are expressed in the placenta in the syncytiotrophoblast during the first trimester of pregnancy
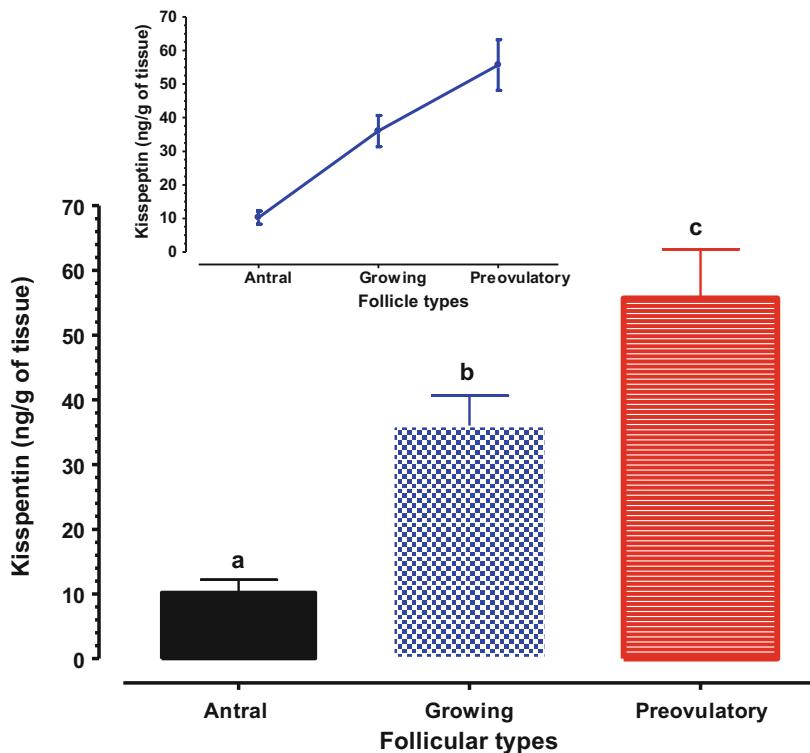
Fig. 3.4 Mean (±SEM) content of kisspeptin in the follicles of different stages of development. A total of 10 g of antral (5–6 mm), growing (8–10 mm), and preovulatory (15–16 mm) follicles was extracted (*n* = 4). Bars indicated with different letters (**a**, **b**, **c**) differ (*p* < 0.001) [Adopted from Mondal et al. 2015]

(Bilban et al. 2004). And, it was seen that the decreased kisspeptin and PIBF (progesterone-induced blocking factor) expressions in trophoblasts and deciduas were associated with recurrent spontaneous abortion (RSA), because expressions of these molecules in syncytiotrophoblasts and cytotrophoblasts were decreased in RSA women than controls (Wu et al. 2014). Moreover, reduced trophoblast invasiveness was associated with the development of preeclampsia as there were lower circulating serum kisspeptin concentrations in women pregnancies complicated by preeclampsia than in healthy pregnancies (Matjila et al. 2016).

In bovines, it was seen that kisspeptin-1r/kisspeptin system plays a crucial role during pregnancy by regulating the proliferation of cells of bovine placental cotyledon cell lines isolated during the first trimester of pregnancy, but it was not involved in modulating placental progesterone secretion during this stage of pregnancy (Martino et al. 2015). Mondal et al. (2016b) reported higher plasma kisspeptin levels in early pregnant than nonpregnant mithun (*Bos frontalis*) cows. Plasma kisspeptin concentrations were found to increase as the pregnancy advances in crossbred cows (Mondal et al. 2015; Fig. 3.6) and placental kisspeptin levels were also found to
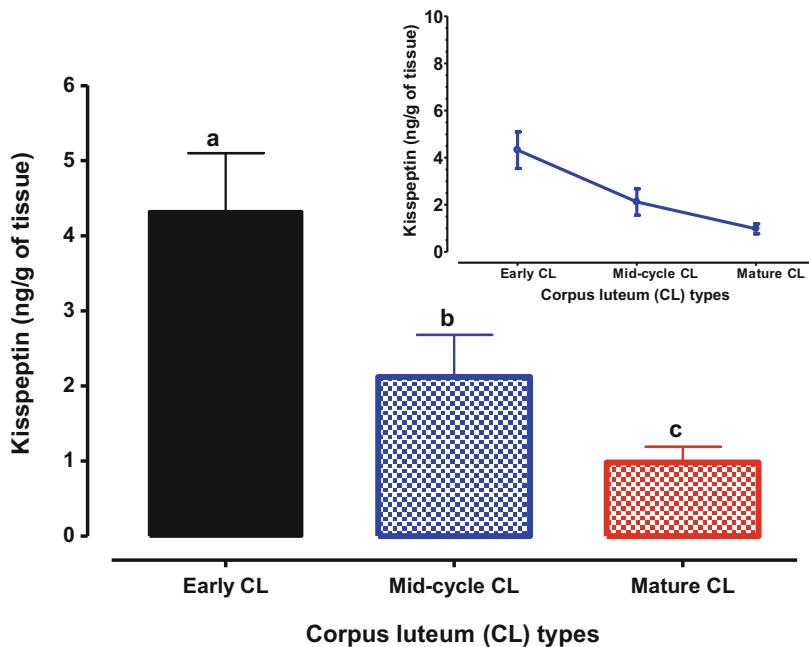
Fig. 3.5 Mean (±SEM) content of kisspeptin in the bovine corpus luteum (CL) during different stages of development. A total of 10 g of early cycle, midcycle, and mature CL tissues was extracted ($n = 4$). Bars indicated with different letters (**a**, **b**, **c**) differ ($p < 0.001$)

increase with the advancement of bovine pregnancy (Mondal et al. 2015; Fig. 3.7). And in small ruminants, KiSS-1 gene affected litter size in goats, because it was seen that the single nucleotide polymorphism in goat KiSS-1 gene had significant effects on litter size (Hou et al. 2011).

3.6 Conclusions

During the first decade of kisspeptin discovery, it is interesting to underline its revolutionary characteristics that have registered the kisspeptin-KiSS1/KiSS1R system in the top priority of research in a variety of mammalian species including livestock. Its multifaceted functions have been proved beyond doubt: from its classical function as a melanoma suppressor agent to a potential gatekeeper of the hypothalamo-pituitary-gonadal axis. Kisspeptin controls pubertal onset, follicular development, emergence of follicular waves, and ovulation. There are also evidences that support its active participation in the autocrine/paracrine regulation of ovarian function, early embryonic development, implantation, placentation, and maintenance of pregnancy. Though most of the research has been conducted in rat and human models, a good quantum of work has already been initiated in livestock species, which clearly showed that kisspeptin is a potent secretagogue of GnRH and
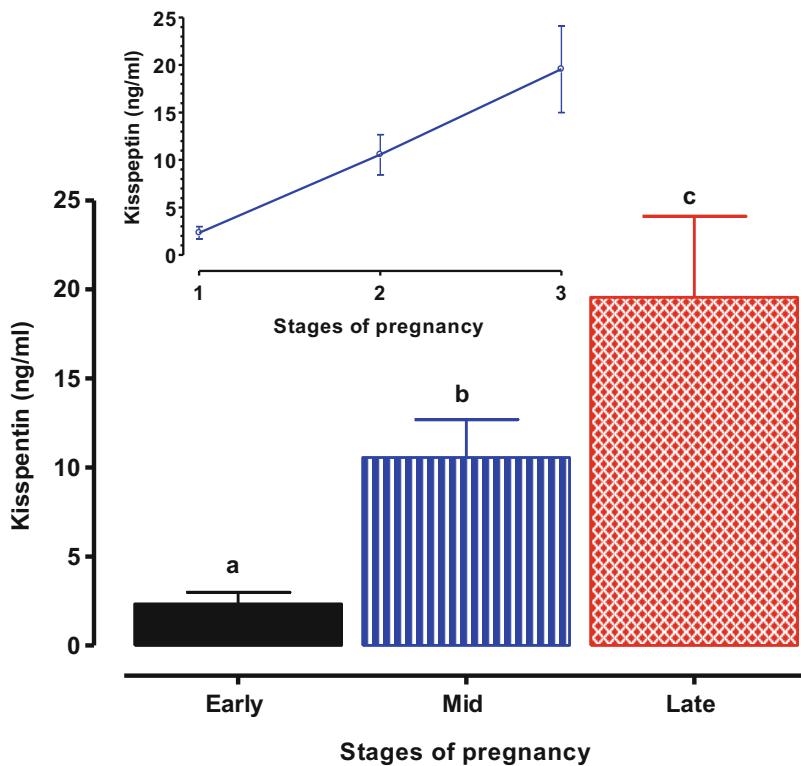
Fig. 3.6 Mean (±SEM) profiles of blood kisspeptin in pregnant cows during different stages of pregnancy viz., early (<10 weeks; $n = 6$), mid (>10 to <20 weeks; $n = 6$), and late (>20 to 36 weeks; $n = 6$) stages of pregnancy. Bars indicated with different letters (**a**, **b**, **c**) differ ($p < 0.001$). [Adopted from Mondal et al. 2015]

can potentially be used in assisted reproduction. It can be used to induce puberty in prepubertal animals. Similarly, kisspeptin can be used as a wonder molecule for estrus synchronization protocol for timed AI. It can also be used as an ovulation inducer in anovulatory cases and for enhancing follicular growth. Additionally, kisspeptin holds promise in the field of in vitro maturation (IVM) of oocytes, in vitro fertilization (IVF), and in vitro culture (IVC) of embryos. Furthermore, use of recently available kisspeptin analogs like C6 that have an extended half-life and high biological potency can be used for fertility management in livestock species in general and bovine in particular. A lot of research is needed to be undertaken in the field of kisspeptin biology in livestock species particularly the kisspeptin analogs and its role in embryo implantation, placentation, IVM, IVF, IVC, etc. It is interesting to note that the research in the field of kisspeptin biology, which was started with its metastasis suppressor function, has now been reached to reproduction as an stimulator of GnRH, and still we have to travel a lot to unravel its role in the assisted reproduction particularly embryo survival and IVF.
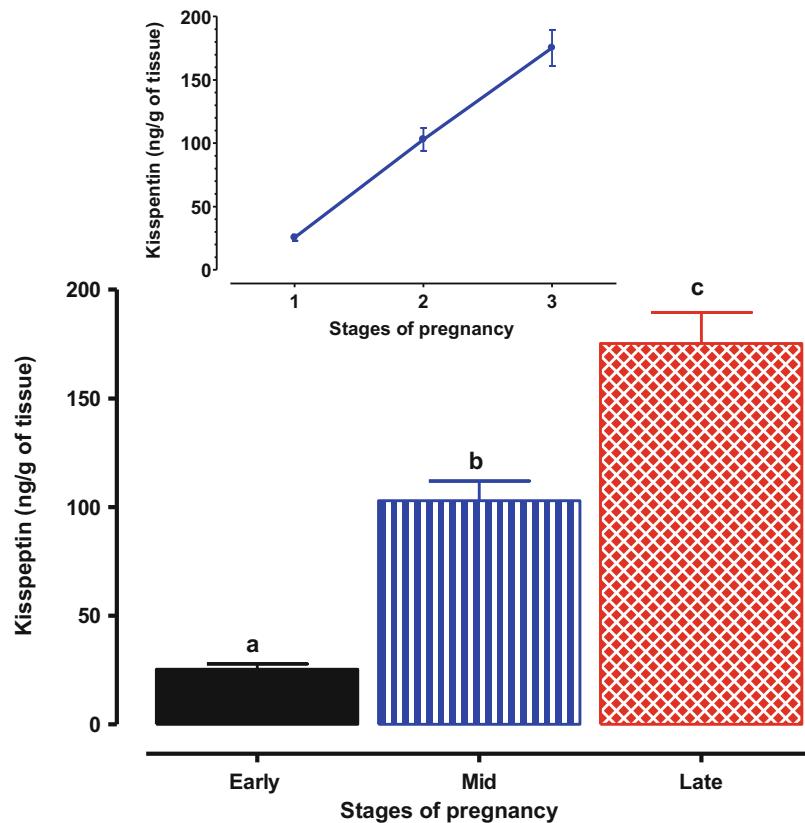
Fig. 3.7 Mean (±SEM) content of kisspeptin in bovine placental tissues collected from animals of different stages of pregnancy. A total of 10 g of placental tissues was extracted ($n = 6$). Bars indicated with different letters (**a**, **b**, **c**) differ ($p < 0.001$). [Adopted from Mondal et al. 2015]

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Nutritional Strategies to Improve Reproductive Efficiency in Cattle and Buffaloes

4

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Abstract

Nutrition is an integral part of biological system. Excess or deficiency of any nutrient will affect availability of other nutrients and hamper metabolic process. Energy, protein and minerals are essential for optimum reproduction. Improved genetic potential for milk production causes stress on animals and consequently reduces fertility. Negative energy balance is crucial factor for delayed fertility in lactating dairy animals. Demand for protein increases with peak milk yield causes excess supply of protein, in particular, rumen degradable protein (RDP), leading to infertility or repeat breeding in high yielders. Requirement of minerals may be lesser in quantity, but it exerts huge biochemical role viz., integral part of several enzymes, synthesis of hormones, antioxidant, maintenances of epithelium, etc. This chapter deals with nutrients needed for efficient reproduction, its function, level of feeding and feeding strategies.

Keywords

Dairy cattle · Buffaloes · Nutrition · Reproduction · Energy · Protein · Minerals

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4.1 Introduction

During the last few decades, milk production increased dramatically, but fertility rate has not been maintained well. Improved production potential through cross breeding programme increases production stress in dairy animals which affects the conception rate. Nutrients are drained during milk production, creating increased nutrient demand for reproduction. In India, various feeding systems are being followed in different agro-climatic conditions due to variations in the availability of feeds and fodder. In peri-urban areas, dairy animals are reared only for milk production and sold off after drying. Those animals are fed excess quantity of protein-rich diet for higher milk production which causes repeat breeding. On the other hand, in rural areas, animals are fed locally available feeds and fodder comprising primarily of crop residues, which are low in nutrients and digestibility. There are multitude of factors affecting reproduction of dairy animals, which include shortage of feeds and fodder, lack of few nutrients in the diet, improper feeding, excess feeding in peri-urban dairies and organized farm, breed variation, species variation, climatic condition, availability of insemination services, and lack of awareness of farmers on feeding and reproductive behaviour of dairy animals (Benon et al., 2015). While maintaining the reproductive efficiency in dairy animals, care must be taken for adequate feeing at the right time in one hand and also over come production stress in another. Reproductive behaviour of cattle and buffaloes varies, latter shows silent oestrous which is unnoticed by the farmer. Energy, protein and minerals, especially trace minerals and vitamins, play a major role in animal reproduction. The major function of nutrients on reproduction is illustrated in Table 4.1. To know about newer approaches like nanotechnology and its application is essential to improve reproduction. In this chapter, nutrients needed for efficient reproduction, its function, level of feeding and feeding strategies will be discussed in detail.

4.2 Energy

Energy is the most important critical factor for production and reproduction in lactating dairy animals. Reproduction of the postpartum animal and early oestrous sign mainly depends on management of negative energy balance during early lactation. Hence, the energy is not only important for peak milk production and also significantly contributes to reproductive efficiency of the herd.

Energy is contributed by group of nutrients viz., carbohydrate, protein and fat. Protein is mainly involved in bodybuilding and production purpose rather than energy conversion. Both soluble and insoluble carbohydrate contributes most of the energy required to ruminant animals through rumen fermentation and production of volatile fatty acid and also some extent, energy contributed by fat. Volatile fatty acids play major role in energy metabolism in lactating dairy animals, it accounts for more than 70% of the total energy requirement. Glucogenic fatty acid (propionic acid) contributes more than 80% of required glucose for milk sugar synthesis. Apart from energy, major limiting nutrient for peak milk production is protein. High milk

Table 4.1 Functions of nutrients on reproduction

Nutrients	Reproductive function	Reference
Energy	• Growth and development of reproductive organs • Deficiency prolonged postpartum anoestrus in cattle and buffaloes • Resumption of ovarian activity • Development of gonadal organs • Hormone synthesis and release	Lohakare et al. (2012) Peter et al. (2009), Kumar et al. (2013) Beam and Butler (1999) Beam and Butler (1998) Santos (2008)
Protein	• Delayed puberty • Cessation of oestrus cycle • Excess protein—low conception rate	Kaur and Arora (1995) Agrawal (2003) Westwood et al. (1998)
Calcium	Uterine muscle contraction, uterine involution, maintain uterine muscle tone	Alotaibi (2014)
Phosphorus	Maintenance of oestrus cycle	McClure (1994)
Zinc	Wear and tear and of uterine epithelium Maintenance of semen quality	Burtis et al. (2006) Swain et al. (2016)
Copper	With zinc—Reproductive hormones—Progesterone and oestrodiol	Roychoudhury et al. (2016)
Manganese	Synthesis of steroid, oestrogen, progesterone and testosterone	Keen and Zidenberg-Cheer (1990)
Iodine	Reduces fertility in male and female Maintaining semen quality	NRC (2001) Rajendran et al. (2002)
Selenium	Role as antioxidant Maintain health of uterine tissue, ovary	Farahavar et al. (2020) Singh et al. (2019)
Cobalt	Attain puberty, uterine involution Maintain early pregnancy	Satish Kumar (2003)
Boron	Increased sperm output, sperm motility Enhanced immune and antioxidant capacity	Binsila et al. (2019)

production during peak milk production period causes negative energy balance, this leads to series of changes like weight loss and metabolic and hormonal changes (Wankhade et al., 2018). It can be well explained in lactation curve as shown in Fig. 4.1.

Milk production will increase continuously up to 60 days post calving. Peak milk production in crossbred dairy cow is expected at 60 days to 90 days of post calving. During milk production, all nutrients are drained to synthesize milk constituent. DMI usually peaks around 150 days to 180 days. The gap between calving to peak DMI period is crucial for dairy animal for maintaining health, production and reproduction. The nutrients may not be sufficient enough from DMI during peak milk production and it is mostly coming from reserve. Normally negative energy balance occurs at 50 to 90 days of early lactation. The reason behind negative energy balance is mainly insufficient dry matter intake (DMI) and associated sudden peak milk production and its mismatch. Hence, sudden demand for nutrients required for milk production is compensated from body reserve. Body reserve is used up to 100–120 days of lactation period for milk production. During this period, body weight will reduce and afterwards body weight will be maintained for a certain
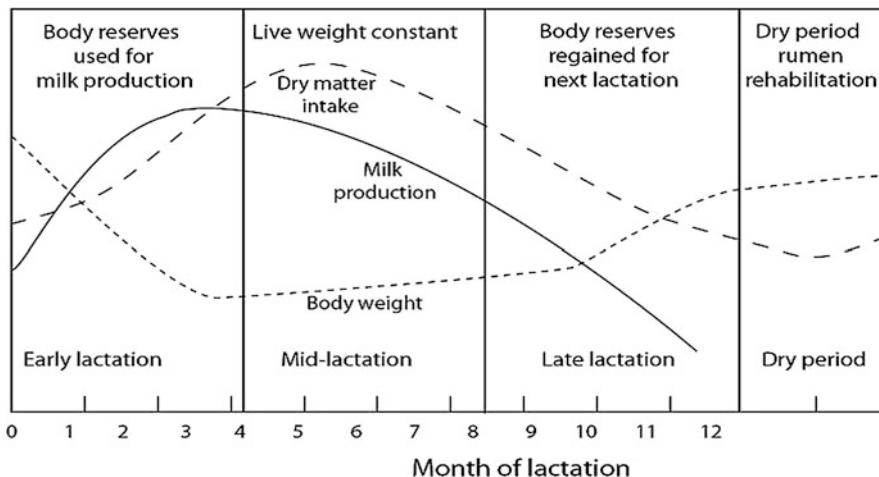
Fig. 4.1 Milk yield, Dry matter intake and live weight changes during lactation cycle

period from 120 days to 240 days and then slowly regain if DMI increases steadily. However, body reserve can supply limited quantities of nutrients leading to shortage of nutrient, which may affect milk production or some time leading to metabolic disorder like ketosis and milk fever. Hence, conditioning of animal during dry periods is essential for optimum milk production and reproduction and to avoid metabolic disorder. As per the available literature report, over conditioning leading to risk of milk fever, ketosis and lower DMI than moderately conditioned dairy cow and buffaloes.

Hence, energy requirement is more during early lactation with physiologically lower DMI. Increased demand can partially be met by feeding high-density energy feed rather than roughage-rich diet. Based on production and DMI, feed should vary with nutrient density especially energy density. Phase feeding is the correct option for varying nutrient demand and feed formulation also varies with requirement and feed intake. During close-up period, DMI will reduce dramatically and density should be increased in feed. In fresh cow, DMI will not increase as compared to milk production, ultimately nutrient density should be increased further to meet nearer to demand of nutrient for milk production. During negative energy status, there will be increased lipolysis with more ketone bodies, low glucose and insulin level in blood. This metabolic shift is the main cause for suppression of increase in luteinizing hormone (LH) pulse frequency that is vital for growth of follicles. Low glucose and insulin levels are the primary signals influencing the release of gonadotrophin-releasing hormones (GnRHs) at central nervous system (Fig. 4.2). Research suggested that inverse relation exist between days of first ovulation, fertility and energy balance during the period of first 3 weeks of early lactation. Similarly high producing animals show lower conception rates at first service. Longer post-partum anoestrous period is noticed in buffaloes than crossbred cow
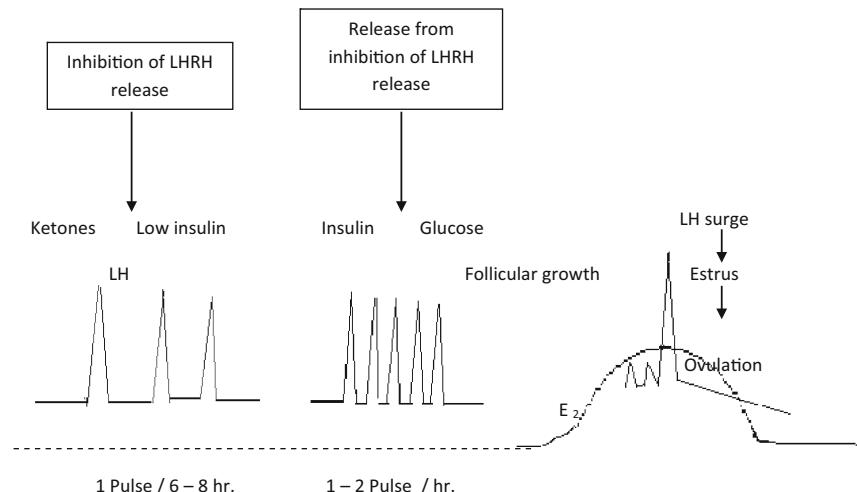
Fig. 4.2 Endocrine events in low/adequate energy status

in India. In addition, those animals having more days in negative energy balance have more days of post-partum anoestrous.

Partitioning of energy for production and reproduction is crucial during maintenance of pregnancy. In beef-producing animals, energy is channelized to the development of muscle mass along with adipose tissue, whereas in dairy animals, it is channelized towards milk constituent synthesis like lactose, milk fat and protein and secretion of milk. Energy is required for synthesis of most of organic constituent of milk.

Energy requirement varies with growing adult and lactating animals. Maintenance energy requirements for indigenous animals vary between 61 and 104 kcal ME/ kg metabolic body weight ($W^{0.75}$) in dry cows and between113 and 160 kcal ME/ kg $W^{0.75}$ during lactation (ICAR, 2013). ME requirement for lactating Haryana cows and crossbred cows (Brown Swiss X Sahiwal) was estimated to be 127 and 131 kcal/ kg$W^{0.75}$, respectively (Patle and Mudgal, 1975). Kearl (1982) adopted the value of 118 kcal/ kg$W^{0.75}$ for estimating the nutrient requirements of cattle against 125 kcal/ kg$W^{0.75}$ for buffaloes. Values of ME requirements for maintenance in buffaloes ranged between 121 and 178 kcal/ kg$W^{0.75}$ (Sivaiah and Mudgal 1983). A value of 128 kcal/kg$W^{0.75}$ was adopted as the ME requirements value while recommending the nutrient requirements of buffaloes (Paul and Lal, 2010). Based on the earlier report, ICAR (2013) adopted the value of energy requirement for maintenance to be equivalent to 122 kcal ME/kg $W^{0.75}$ both for cattle and buffaloes. However the growing animal, energy requirement is differ.

Milk-producing animal energy requirement is calculated based on milk production. Energy is required for milk in addition to maintenance requirement. Several workers have reported different values ranging from 1039 to 1188 Kcal ME/Kg FCM (Patel and Mudgal 1976; Ranjhan 1998). ICAR (2013) adopted energy

requirement per kg of FCM is 330 g and 360 g of TDN in cattle and buffalo respectively.

4.3   Strategies on Energy Requirement

Impaired energy balance in high producing animals is very difficult to overcome. Maintaining the energy intake at close-up cow (21 days prior to calving) and increasing intake rapidly thereafter in fresh cow (calving to 21 days post calving) should be ensured. Recommended nutrient levels were calculated using TMR – Maker software developed at ICAR-NIANP is illustrated in the Table 4.2.

As illustrated above, to increase energy density of the feed, fat is supplemented in the diet. The concentrate is to forage ratio increased in fresh cow diet to meet the TDN requirement of total mixed ration up to 70–75%. The crude protein, calcium, phosphorus content of the fresh cow diet is also increased to meet all these required nutrients for milk production. Supplementation of cereal grain like crushed ragi grain as energy source (@ 1 kg per day) for 90 days to crossbred dairy cows during early to mid lactation period reduced the milk urea nitrogen level, indicating a positive effect on energy utilization (Gowda et al., 2009). In small farms, availability of feed and fodder is major concern and it seems to the important factor to overcome negative energy balance. Prepared feed should be more palatable to ensure high dry mater intake apart from denser diet. To increase DMI, the animal should be allowed ad lib feeding and ensure availability of feed in the manger most of the time. Mature buffaloes maintained on a negative energy balance (NEB) diet had suppressed ovarian follicular turnover after 60 days of treatment and a decline in oocyte quality after 80 days (Campanile et al. 2010). During the period of negative energy balance, the blood concentrations of non-esterified fatty acids (NEFAs) increase, and the insulin-like growth factor-I (IGF-I), glucose and insulin are low. Selvaraju et al. (2002) reported that there is beneficial effect of insulin treatment on fertility in repeat breeding cattle. Studies evaluating the effects of supplemental fat on reproductive performance of buffaloes are limited; however, the recent report (Campanile et al., 2010) suggested that buffalo heifers may potentially have the capacity to undergo metabolic adjustment and reduce their energy requirements when dietary energy is limiting (Table 4.3).

4.4   By Pass Fat

In early lactating high producing animals, energy demand is enormous and increasing density of feed by supplementing fat up to a certain extent may be possible. If level of fat (ether extract) exceeds 5% of the diet, it may lead to negative effects on cellulolytic bacteria and in turn cause decreased fibre fermentation and production of acetic acid in the rumen. Acetic acid is the precursor for milk fat synthesis; reduction in this VFA leads to depression of milk fat. Hence, to avoid suppression of fibre fermentation, supplemented fat may be added in the form of protected fat (bypass

Table 4.2 Recommended nutrients levels and illustrated TMR composition for feeding of cow pre-, post-21 days of parturition, early, mid and late lactation period

Feeding stage	Details	Recommended Nutrients in diet	Illustrated TMR composition[a] for 100 kg diet (as fed basis)
Close-up cow	21 days prior to calving	CP: 15–16% TDN = 65–67% NDF = 35–40% NFC = 30–35% Fat = 4% Ca = 0.7% P = 0.3% DMI: 1.25–2.0%	Green fodder: 71 kg Dry fodder: 2 kg Con. Mix: 25.2 kg Fat/oil: 1.3 Salt: 0.13 kg MM: 0.37 kg Calculated value: CP: 15%;TDN; 65% Ca: 0.7%; P: 0.48% DM:40.5% *Note: For 450 kg cow 23–25 kg TMR is sufficient*
Fresh cow	From calving to 21 days post calving	CP: 18% TDN = 70–75% NDF = 30–40% NFC = 35–35% Fat = 5% Ca = 1.1% P = 0.5% DMI: 2.0–2.5%	Green fodder: 65 kg Dry fodder: 1 kg Con. Mix: 4 kg Maize 10 kg Soya DOC 9.5 kg Cotton DOC 6 kg Fat/oil: 3.3 kg Calcite 0.23 kg Salt: 0.13 kg MM: 0.84 kg Calculated value: CP: 18%;TDN; 73% Ca: 1.1%;P: 0.77% DM:44.8% *Note: For 450 kg with 15 L milk yield cow 35–38 kg TMR is sufficient*
Early lactation cow	Early lactating cow (21–100 days)	CP: 17–18% TDN: 70–75% NDF: 30–35% NFC:30–40% Fat: 5–6% Ca:0.8–1.1% P: 0.5–0.9% DMI: 3.5–4	Green fodder: 65 kg Dry fodder: 1 kg Con. Mix: 6.5 kg Maize 10 kg Soya DOC 7 kg Cotton DOC 6.5 kg Fat/oil: 3.3 kg Calcite 0.23 kg Salt: 0.13 kg MM: 0.85 kg CP: 17%; TDN; 73% Ca: 1.1%;P: 0.74% DM:44.8% *Note: For 450 kg with 20 L milk yield cow 38–40 kg TMR is sufficient*
Mid lactation cow	Mid lactating cow (101–200 days)	CP: 15–16% TDN: 65–70% NDF: 30–38% NFC:30–44%	Dry fodder: 3 kg Green fodder: 63 Con. Mix: 9.2 kg Maize 9.6 kg

(continued)

Table 4.2 (continued)

Feeding stage	Details	Recommended Nutrients in diet	Illustrated TMR composition[a] for 100 kg diet (as fed basis)
		Fat: 4–6% Ca:0.8–1.0% P: 0.4–0.8% DMI: 3.0–3.5%	Soya DOC 2.0 kg Cotton DOC 8.6 kg Fat/oil: 2.7 kg Salt: 0.14 kg MM: 1.0 kg Calculated value: CP: 15.2% TDN; 70% Ca: 1.0% P: 0.73% DM:45.5% *Note: For 450 kg with 15 L milk yield cow 30–35 kg TMR is sufficient*
Late lactation	Late lactation (201–305 day)	CP: 13–15% TDN: 60–65% NDF: 33–43% NFC:30–45% Fat: 3–5% Ca:0.7–0.9% P: 0.4–0.7% DMI: 2.8–3.3%	Dry fodder: 5.5 kg Green fodder: 58.8 Con. mix: 10.4 kg Maize 1.5 kg Cotton DOC 0.5 kg Wheat bran:12.5 Gram chunnies10.5 Calcite:0.19 kg Salt: 0.15 kg MM: 0.42 kg Calculated value: CP: 13%;TDN; 61.5% Ca: 0.9%;P: 0.45% DM:47% *Note: For 450 kg with 15 L milk yield cow 28–30 kg TMR is sufficient*

[a] Calculated in NIANP—TMR—Maker software.

rumen digestion). This protected fat digested in the true stomach and subsequent gastrointestinal tract, which meet the energy demand of the animals and also improved milk fat content in the milk and improve reproductive performance, because fatty acids are needed for steroid hormone synthesis. Protected fat supplementation to cows maintained on natural feeding practices at field condition improved the milk production and reproductive efficiency in dairy cattle (Gowda et al., 2013a). For cattle, 20 g bypass fat per kg milk yield and for buffalo 15 g per kg milk yield can mixed in concentrate mixture and fed. However, the total quantity should not exceed 200 g for cows and 150 g for buffaloes, to prevent adverse effect of residual-free fat on rumen fermentation.

Table 4.3 Suggested composition of concentrate mixture for different categories of cows

Ingredients (%)	Level of milk production (kg)			
	Low (5–10 kg)	Moderate to medium (15–20 kg)	High (20–25 kg)	Very high (elite category) (>25 kg)
Grains	18–20	28–30	38–40	45–50
Wheat bran	30–35	20–25	18–20	6–8
Oil cakes	15–18	22–25	28–30	32–35
Rice polish/rice bran	15–20	10–15	5–8	3–5
Gram husk	8–12	5–8	3–5	–
Molasses	5	5–7	8–10	10
Urea	0.50	1	1	1
Mineral mix.	1	2	2	2
Common salt	0.5	0.5	1	1
Calcite	–	–	1	2
Sodium bicarbonate	–	–	0.5	1
Bypass fat	–	–	1	1.5
Vitamin premix	–	–	0.1	0.1
Chelated trace min.	–	–	0.01	0.02
CP in con mix. (%)	16–17	18–19	20–22	23–24 (50–55% By pass protein)
TDN in con mix. (%)	62–63	64–65	71–72	73–74

4.5 Protein

4.5.1 Protein Deficiency and Reproduction

In heifer and postpartum cow, nutrition plays a major role in development of ovary, onset of cyclicity, subsequent conception rate, and embryonic and foetal growth. Protein rather excess causes more damages than deficit. Protein is required for genital organ development, puberty and synthesis of sex hormone. In commercial farms, high plane of nutrition may lead to excess protein consumption which in turn causes reduced fertility.

In female calves, attaining target weight is essential for puberty and subsequent heat symptom. Puberty is based on the growth rate of the animals. In buffaloes puberty attain when animal reaches 60% of its body weight (Warriach et al., 2015). It depends on birth weight, feeding of calves at an early growth stage and amount of nutrient especially protein present in calf starter and subsequent grower feed. Hence,

rapid achievement of growth and body size leads to early puberty and reduces first calving time in heifers (Kaur and Arora, 1989). In tropical countries, puberty is delayed in grazing cattle and buffaloes due to lack of protein content in grazing land especially in fallow grazing land (Rajendran and Balakrishnan, 2012).

Deficiency of protein or alteration of energy protein ratio resulted in delay in oestrous cyclicity. Loss of body weight at postpartum at the extent of 15% can lead to infertility in dairy animals. Period of anoestrus is increased from 21.8 days to 159.8 days in buffaloes fed optimum and low level of protein respectively (Kaur and Arora, 1989) and in cattle, it is increased from 36.3 days to 170.3 days (Juneja and Arora 1990).

High plane of nutrition has increased ovulation rate (Downing et al., 1995) in ewes. It is well observed in ewes, feeding of high plane of nutrition with high protein content increased multiple ovulation (Demmers et al., 2011).

Though the protein is essential for survival of embryo and foetal growth, it can be obtained from the body reserve instead of dietary sources. Low protein diet may not have a deleterious effect on conception rate and foetal growth in early stage, but at later stage affect birth weight (Bell et al., 1992).

4.5.2   High Protein Diet and Reproduction

High protein diet and its effects on follicle development, oocytes and embryos have been well documented (Butler, 1998). High protein diet enters the rumen: there it is converted into ammonia by rumen microbes and is then finally converted into microbial protein. During this process, increased ammonia concentration in blood, elevate blood urea nitrogen (BUN) (Laven et al., 2007; Sinclair et al. 2014). Butler (1998) in his review concluded that excessive rumen degradable protein (RDP) or rumen undegradable protein (RUP) could be cause for lower conception rate. Meta-analysis to find out by the effect of dietary CP on pregnancy rate by Lean et al. (2012) indicated an overall 9% ($P = 0.019$) reduction in chance of conception in cows fed diets containing higher or more degradable CP within a dietary CP range of 11% to 23%. Aboozar et al. (2012) reported feeding of high level of RUP improved range of reproductive traits in cows.

Most of the studies in recent past suggested that high protein content is deleterious to fertility. However, very few studies have reported on whether low protein diet improves fertility or not. Dietary CP given below 15% reduced milk yield and reduced negative energy balance (Law et al., 2009) and decreased days open from 86.6 days to 79.2. Hence, more than 16% CP level in the diet may be detrimental on reproductive traits in dairy cattle (Sinclair et al., 2014). Cows fed 20–23% CP diets (as compared to 12–15% CP) had decreased uterine pH, increased blood urea and altered uterine fluid composition (Jordan et al., 1983; Elrod and Butler, 1993). However, the optimum CP content of the diet 14–16% is sufficient to maintain milk production as well as fertility of dairy cow.

4.5.3   Strategies on Protein Requirement

Excess protein is more deleterious to fertility of the animal than normal or slightly less protein diet. As discussed early, excess rumen degradable protein releases ammonia in rumen, most part is utilized for microbial protein synthesis and remaining causes high blood urea nitrogen as shown in Fig. 4.3. BUN is considered as reliable biomarker to assess excess protein intake. BUN concentration exceeds 20 mg/dl in lactating cows and greatly affects conception rate. (Ferguson et al., 1988). Hence, phase feeding of animal is essential instead of same diet composition. In phase feeding, nutrient density is changed according to the phase of production. Phase feeding starts from close-up cow (21 days prior to calving), fresh cow (0–21 days post calving), early lactation cow (21–100 days), Mid-lactation cow (100–200 days) late lactation cow (200–305 days) as shown in illustration of feed formulation in Table 4.2. It can be well observed that protein (%) content of the diet is decreasing according to the milk yield. The crude protein content of the diet varies and starts in fresh cow as high as 18% and in late lactation as low as 13–15%. Protein content in early lactation diet is quiet important, because it has to balance milk yield as well as conception rate. Animals conceiving at early lactation will be considered as best herd than at mid-lactation period.
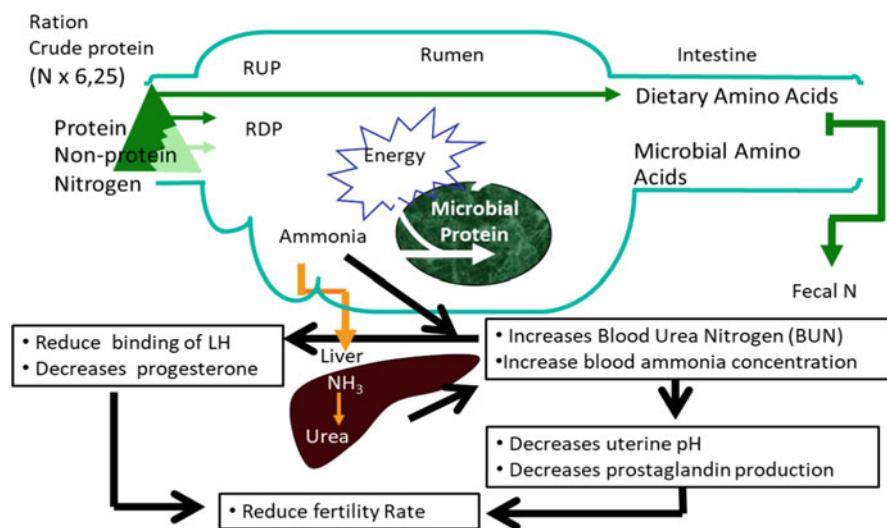
Fig. 4.3 Schematic representation of excess protein on reproduction as described in literature (Butler, 1998; Barton, 1996; NRC 2001)

4.5.4   Supplementation of Bypass Protein

As per NRC (2001) requirement for RUP is more than 2.6% in the diet of cattle for yielding10 kg fat corrected milk and its requirement up to 12.1% for high yielding dairy animals. Hence, it is desirable to incorporate protein sources which are rich in bypass protein in the concentrate mixtures, especially during the early lactation. Commonly available protein sources are also having bypass protein which include cotton seed cake, solvent-extracted coconut cake, maize gluten meal and brewer's grain. With formaldehyde treatment (1 g/100 g of protein, i.e., 10.5 g formalin mixed with 1 kg groundnut cake, kept in airtight containers for a week period), bypass protein can be prepared. As per NRC (2001), it is necessary to maintain a minimum of 4–12% of RUP in the diet (it is 45–50% bypass protein in the total crude protein) of the high yielding animal. Feeding of bypass protein has shown a positive effect on milk yield, milk quality and for early conception (Tandon et al., 2008). Protected amino acids in encapsulated form (methionine, lysine, histidine) are recommended for high producing animals, about 50–100 g/animal depending on milk yield and this will reduce the quantity of cake (ground nut cake/soybean meal/sesame cake) by about 400–450 g/animal. Tandon et al. (2008) reported that feeding of bypass protein in cow reduces number of service per conception.

4.6   Minerals

Minerals play a vital role in the livestock industry. Minerals perform biochemical, digestive, skeleton building, biosynthesis process, growth and reproductive process of animals (McDowell, 2003). Role of minerals in reproduction has been reviewed by Gowda et al. (2013b). Minerals are acting as co-factors for enzymes in most of biochemical reactions including metabolism and synthesis of hormones or even antioxidant activities. Some of the minerals are essential for the mucous membrane integrity and normal secretary function of epithelial cells of reproductive organs. Few minerals are involved in ova development, maturation and release. Not only individual minerals have been playing a role in biochemical processes, but also in combination. However, excess level of one mineral also affects the availability of others. Hence, the bioavailability of a mineral from a particular source is determined in relation to its functional availability from a standard source. Use of a standard source allows expression of bioavailability in terms of relative biological availability (Ammerman et al., 1995). Several technologies are adopted to increase the bioavailability of the mineral, chelated mineral, nanomineral and supply through individual minerals in the form of sulphate source, encapsulated minerals are the few of them.

4.6.1   Chelated Minerals

Inorganic minerals bound to organic moiety (ligand) are called chelated minerals. Non-metallic ligands are present in chelated mineral; hence, it is organic. Atoms,

which are able to donate their electrons, are called donor atoms. Based on number of donor atom, it may be called as monodent (one atom donor) or polydents (more than two atom donor). Polydents can form chelated minerals, since they can bind a metal within their electronic dents or claws (Chele means claw in Greek). Several studies have been carried out on amino acid as ligand in the chelated mineral, but chelated minerals are also produced with polysaccharides or high molecular weight ligands.

Studies observed that organic trace minerals improved immune response in dairy cows and calves, and also improved milk yield and reproductive performance. Pal et al. (2010) reported that the bioavailability of copper (Cu) and zinc (Zn) and their accumulation in tissues were much higher in ewes when supplemented with Cu and Zn from organic sources than inorganic sources. Supplementation of zinc with glycine chelate improves the growth performance of pigs (Wang et al., 2010) and broiler birds (Feng et al., 2009). Due to higher bioavailability of organic trace minerals, Nollet et al. (2008) and Bao et al. (2007) suggested that broiler performance and concentration of minerals in tissues could be achieved by supplementing lesser minerals from organic sources. Peters and Mahan (2008), demonstrated that supplementing organic trace minerals in sow diet had improved sow reproductive performance.

4.6.2 Nanominerals

Nanominerals improve bioavailability due to the increase in the surface area as shown in Fig. 4.4 (Rajendran, 2013). Further, nano-encapsulation technique avoids mineral-mineral or mineral-nutrient interaction and enhances their utilization at target sites. Recently the use of nanomineral reduces (Swain et al. 2018b) its requirement up to 50% of its recommendation (ICAR, 2013). Nano-Zn has been
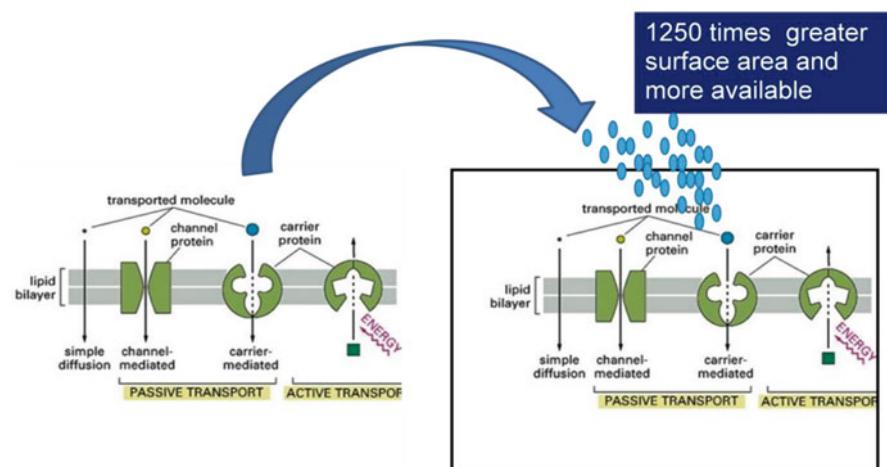

Fig. 4.4 Nanoparticle absorption is high due to increase in surface area (Rajendran, 2013)

reported to reduce the somatic cell count (SCC) in subclinical mastitis cow and improved mastitis conditioned with increase in milk production than other conventional ZnO sources (Rajendran et al. 2013). Supplementation of ZnO NPs had improved milk production in subclinical mastitis animal; thus, it can be used as a preventive as well as a curative agent. ZnO NP has been reported to enhance growth performance, improve the feed utility and provide good economical profit in weanling piglets (Yang and Sun 2006) and poultry (Mishra et al. 2014). Mishra et al. (2014) observed an improved growth rate in layer chicks than inorganic Zn supplemented groups due to supplementation of nano-Zn at much reduced doses. Many researchers have pointed out the antimicrobial action of metal oxide NPs. ZnO NPs have bactericidal effects on both Gram-positive and Gram-negative bacteria (Arabi et al. 2012) and it has the potential in reducing bacterial growth for practical applications in animal without the fear of antibiotic resistance and residues. Swain et al. (2018a) reported nano-zinc improves fibre degradability even at half dose of ICAR (2013) recommended level. The improved bioavailability of nano-zinc might be due to higher surface area as shown in Fig. 4.4. Nanomineral can be prepared by physical, chemical and biological methods (Swain et al., 2015).

4.6.3 Calcium and Phosphorous

Great demand of Ca is noticed during early lactation for milk production. Calcium homeostasis exerts its function and maintains blood calcium levels in the normal range (9 to10 mg/dl). Initiation of lactation is one of the greatest stresses on Ca homeostasis and is associated with milk fever among high producing dairy cows and buffaloes. Hypocalcaemia also increases the incidence of dystokia, retained placenta, metritis, prolapsed uterus and delayed uterine involution (ICAR, 2013). During the dry period, Ca requirement is 10–12 g/d (foetal and endogenous Ca); hence, mechanism for replenishing plasma Ca is relatively inactive. Upon parturition, Ca requirements are 10 times more than the supply in bloodstream. Feeding a diet containing anionic salts is effective for the prevention of hypocalcaemia, minimizing the occurrence of milk fever and associated reproductive problems. However, excess Ca in the diet may impair reproductive function by causing secondary deficiency of P, K, Mg, Zn, Cu and other trace minerals by inhibiting their absorption in the intestine. As per NRC (2001), the level of calcium and phosphorus should be maintained at 0.65–0.8% and 0.32–0.44%, respectively, in the diet of lactating animals. Calcium and phosphorus ratio in the diet is also important. It ranges from 1.5 to 1 to 2.0 to 1 (Ca:P). Ratio is needed for utilization of those two minerals; however, vitamin D in the diet nullifies the effect of ratio in the diet.

Calcium-dependent mechanisms are involved in steroid biosynthesis in the testis, adrenal glands and ovaries. Calcium plays a role in the utilization of cholesterol by mitochondria or by stimulating the conversion of pregnenolone to progesterone. Phosphorus is often associated with reproductive abnormalities in cattle although infertility due to P deficiency is usually manifested after other signs are readily

apparent. Phosphorus deficiency induces lowered conception rate, irregular oestrus, anoestrus, decreased ovarian activity, increased incidence of cystic follicles and generally depressed fertility. The involvement of P in phospholipid and c-AMP synthesis may be a key to its effect on reproduction.

4.6.4 Zinc and Copper

Zinc deficiency in dairy cows has been postulated to weaken the skin and other stratified epithelia as well as reducing the basal metabolic rate following infectious challenge. Zinc is a co-factor for many proteins and enzymes involved in acute phase response to infection and inflammation. Because the mammary gland is a skin gland, it is likely that zinc will have a positive role in its protection. Skin integrity of the teat has been shown to be specially linked with mastitis prevention. Zinc activates several enzyme systems and is a component of many metalloenzymes. It plays a vital role in hormone secretion, especially related to growth, reproduction, immune-competence and stress. Zinc is also involved in the generation of keratin and in skin nucleic acid and collagen synthesis as well as in the maintenance of normal vitamin A concentration in plasma and in ovarian function. Many animals therefore require supplemental zinc in the diet for normal body function because of either low levels in the dietary ingredients or the presence of antagonistic factors, which decrease the bioavailability of the element. Antagonism might be due to metal-ion interactions such as iron or copper. Source of fibre has also been reported to decrease the availability of zinc. Indian studies have shown that repeat breeding and/or anoestrus conditions in livestock could be controlled by improving Cu and Zn nutrition (Gowda et al., 2013b).

4.6.5 Iodine

The need for iodine for the thyroid activity and reproductive failure often is a secondary manifestation of thyroid dysfunction resulting from iodine deficiency in cattle. Foetal development during iodine deficiency may be arrested at any stage and lead to early embryonic death, foetal resorption, stillbirth or birth of goitrous, weak or dead foetus. Hypothyroidism also can reduce gonadotrophin output by the pituitary. Iodine deficiency in bulls is associated with depressed libido and deterioration of semen quality. Several studies have revealed that supplementation of iodine has improved fertility, reduced stillbirths, abortions and incidence of retained placenta. Infertility in dairy cattle resulting from irregular or suppressed oestrus has been often responsive to iodine therapy. Iodine supplementation is necessary in many areas of deficiency, but toxic amounts of iodine are not favourable.

4.6.6 Manganese

Manganese is involved in the activities of several enzyme systems including hydrolases, kinases, decarboxylases and transferases as well as Fe-containing enzymes which require Mn in their activity. It is therefore involved in carbohydrate, lipid and protein metabolism. It is also needed for bone growth and maintenance of connective and skeletal tissue. Mn also plays a role in reproduction and in immuno-logical function. Mn deficiency results in abnormal skeletal growth, increased fat deposition, reproductive problems and reduced milk production (Gowda et al., 2013a, b).

4.6.7 Selenium and Cobalt

Selenium is a semi-metal that is very similar to sulphur in its chemical properties. It is an essential component of glutathione enzyme system, and a deficiency of selenium will leave the cell vulnerable to oxidation and increase the requirement of vitamin E. It has therefore been usual to supplement in the diets of all classes of animals, because of its antioxidant properties.

4.6.8 Cobalt

Cobalt is an essential trace element in ruminant diets for the production of vitamin B12, which has 4% cobalt in its chemical structure, by the rumen microbes to meet the vitamin B 12 requirements of both the ruminal bacteria and the host animal. This means that a cobalt deficiency is really a vitamin B 12 deficiency that would lead to anaemia.

4.6.9 Chromium

Naturally occurring chromium is crucial for carbohydrate metabolism (Tuormaa, 2000). It is present in high concentration in nuclear proteins; thus, it is necessary for gametogenisis and healthy foetal growth. Chromium plays an important role in the secretion of pregnancy-specific proteins from the uterine endometrium which is helpful in preventing early embryonic death. Chromium significantly improves follicular maturation and LH release. It can possibly lead to lower sperm count and decreased fertility and influences foetal growth and development (Tuormaa, 2000).

4.6.10 Boron

Recently, physiological requirement of the boron was studied at ICAR-NIANP (Binsila et al., 2019; Selvaraju et al., 2017; Gowda et al., 2007), but the role of boron in animal is still not fully understood. Supplementation of boron influences bone growth and maintenance, central nervous system, endocrine and reproductive functions (Nielsen 2017). Dietary boron supplementation in male goats increased the sperm output, sperm motility and enhanced the immunity and antioxidant capacity (Binsila et al., 2019). The improved semen quality can be attributed to enhanced expression of testicular *SERPIN*, a crucial protein for the regulation of spermatogenesis process (Binislla et al., 2019).

4.7   Strategies on Mineral Requirement

Sufficient mineral supply is crucial for optimum reproduction. It starts from close-up cow (21 days prior to calving). Feeding of dietary anion is advised to prevent onset of milk fever and related complication. Reducing DCAD (dietary cation-anion difference) to negative values has been shown by many researchers to prevent this rapid decline in blood calcium at calving. The level should be less than zero postpartum (-10 to -15 meq/100 g dry matter if forages are variable in potassium levels). As illustrated in Table 4.2, Ca level of diet in close-up cow should be lesser than 0.7% and should be increased after calving up to 0.8–1.1% in the diet. There are many anionic salts available as commercial products, but use of ammonium chloride and calcium sulphate would be appropriate. Other sources are $MgSO_4$, NH_3SO_4, MgCl and $CaCl_3$. Similarly phosphorus level should be as low as 0.3% in close-up cow and increased to 0.5% in fresh cow diet and maintained in early lactation and thereafter gradually reduced in mid lactation onwards. Ca, P ratio ranges from 1.5 to 1 to 2.0 to 1 (Ca:P). Ratio is needed for utilization of those two minerals. However, if vitamin D is supplied or synthesized in the body in sufficient quantities (based on expose to sunlight), the ratio of Ca and P may not be that important. Hence, supply of vitamin D up to 20–30 K IU per day is recommended for the lactating animals. Similarly other minerals viz., sodium (0.2–0.45%), chlorine (0.25–0.3%), sulphur (0.20–0.24%), zinc (80 ppm), iron (100 ppm), selenium (0.3 ppm), iodine (0.6–1.4 ppm) Copper (12–30 ppm) manganese (60 ppm) may be supplemented through mineral mixture in the form of inorganic salts or preferably organic trace minerals or in the form of nanominerals (Like zinc, copper and manganese). Supplementation of most limiting minerals in the form of area-specific mineral mixture to dairy cows improved the reproductive performance under field condition (Selvaraju et al. 2009). Minerals may be required in very small quantity, but it exert big role, first it affect luxuries part of life, i.e., fertility. Hence, ensuring the presence of mineral in sufficient quantity may be useful strategy to ensure optimum reproduction in dairy herd.

4.8   Conclusion

Reproduction is a key factor for profitable dairy industry. Improved genetic potential for milk trait negatively correlated with reproduction traits. Moreover, production stress adversely affects reproduction parameters. Proper nutrition plays a major role to improve those negative effects in certain extent, and improve fertility if suggestive nutrition strategies are followed. Negative energy balance and feeding of high protein diet during the early lactation period causes delay in first oestrous and delayed fertility. Hence feeding of transition cow is crucial for both milk yield and reproduction. Energy is the primary reason for reproductive disorder for high milk yielders. High protein diet causes increased BUN level and further sequence of event causes infertility or repeat breeding. Protected fat can improve energy density and also reduces milk fat depression syndrome in early lactating animals. Protected protein supplementation in certain extent is safeguarding for BUN level. Similarly, minerals play a major role in reproduction; extra mineral supplementation causes mineral-mineral interaction causes reduced bio-availability of certain minerals. To improve mineral bio-availability, some techniques are followed like feeding of chelated minerals, nanominerals and encapsulated minerals. Economic supplementation like area-specific mineral mixture also exerts a role in rural area of the Indian subcontinent to improve moderate yielding cattle and buffalo fertility.

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Essential Roles of Metabolic Hormones on Gonadal Functions and Fertility of Livestock

5

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Abstract

Metabolic signals through the hypothalamo-hypophyseal-gonadal axis influence the reproductive functions. Metabolic hormones such as growth hormone, insulin, and insulin-like growth factor-1 (IGF-1) modulate the gonadal function by influencing gametogenesis and steroidogenesis. These growth factors promote the follicular cells mitosis, survival and maturation of the oocytes, ovulation process, and luteal cells function. The effect of these metabolic hormones on male fertility though have not been documented well, addition of IGF-1 as an additive in the semen extender improved semen quality. The metabolic hormones act through their receptors present in the gonads and gametes of various domestic animals. The optimal levels of metabolic hormones in the circulation that are sufficient for manipulating the gonadal function need to be arrived for each of the domestic species. Alteration of metabolic hormones through supplementation of macro- and micronutrients will optimize reproductive efficiency in dairy animals.

Keywords

Metabolic hormones · Growth hormone · Insulin · Insulin-like growth factor-1 · Livestock fertility

The productivity and reproductive capacities of the animals can be improved through modification in traditional methods of breeding, feeding, and management (Walsh et al. 2011). The reproductive axis is controlled by the "metabolic status sensor"

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rather than the "level of nutrition" (Blache et al. 2000). In this regard, the growth factors and metabolic hormones, that is, somatotrophin, insulin, and insulin-like growth factor-I (IGF-I), received considerable attention for improving fertility in domestic animals. Though hypothalamo-pituitary axis is an important site of action for these metabolic signals (Bagath et al. 2016), these factors also act directly on the gonads (Selvaraju et al. 2012a; Dupont et al. 2014). This chapter discusses the recent understanding of the metabolic signals regulating reproductive performance in domestic ruminants.

5.1   Metabolic Hormones and Reproduction

Insulin, the classical metabolic hormone secreted from the β cells of Islets of Langerhans, has been described for its key role in carbohydrate metabolism. Reproductive status, lactation, age, and nutritional status are the major determinants of endogenous insulin concentration in domestic animals. The binding sites for insulin, IGF-I, and GH have been demonstrated in ovary, oviduct, uterus, and embryo. These factors influence the intrafollicular growth (Gong et al. 1994), gametes function (Selvaraju et al. 2010), and embryo development (Xie et al. 2015). Studies directed toward the direct involvement of insulin on reproductive functions showed beneficial effect on gonadal function (Selvaraju et al. 2002, 2003; Baithalu et al. 2013).

5.2   Folliculogenesis

The ovary is one of the sites of action for metabolic hormones in several species. The development of small antral follicles is not strictly gonadotrophin dependent. It is suggested that factors, that is, IGF, FGF, and EGF families, directly influence follicular growth rate by enhancing granulosa cell proliferation. In these follicles, vascularization is poorly developed, suggesting that paracrine regulation might be of particular importance. The growth of small antral follicles under the influence of GH is probably by IGF-I of endocrine origin. The relative importance of different growth factors regulating the growth of small antral follicles has not been well established. The growth factors modulate survival, proliferation, and differentiation of follicular cells, acting in interaction with gonadotrophins (Monniaux et al. 1997) and might act in vivo by enhancing growth and terminal maturation of ovulatory follicles (Rao et al. 2011).

Insulin acts through either its classical receptors or IGF-I receptors or both depending upon the clinical circumstances. The insulin receptors have been demonstrated in the granulosa cells (McArdle et al. 1991). Though the presence of such receptors in the corpus luteum of bovine is unclear, the addition of insulin stimulated progesterone secretion from the buffalo luteal cells cultured in vitro (Baithalu et al. 2013). In a physiological concentration, insulin acts in synergy with FSH and LH through its receptor to stimulate granulosa cell mitosis in small follicles (Gong et al. 1993c; Vinodkumar et al. 2017). Insulin increases

intrafollicular IGF-I production, which is positively correlated with follicular development. In sheep, insulin recruits follicles as well as reduces atresia and thereby increases the number of ovulatory follicles (Matamoros et al. 1991). The peripheral concentrations of insulin and IGF-I were positively correlated with the number of gonadotrophin dependent follicles (Gong et al., 1996). In heifers, administration of long-acting bovine insulin at a concentration of 0.25 IU/kg body weight subcutaneously as a cotreatment with FSH increased the size of the ovulatory follicle (Simpson et al. 1994).

In ruminants, IGF-I stimulates both proliferation and differentiation of granulosa cells in vitro. Depending on species and stage of follicle growth, IGF-I may be synthesized and secreted from the granulosa (Khalid et al. 2000) and thecal cells (Voutilainen et al. 1996). But studies indicate that the intrafollicular IGF-I is derived mostly from the circulating pool (Gong et al. 1993b; Perks et al. 1995). IGF-I receptors are present in the granulosa and thecal cells, and IGF-I acts on the granulosa cells in an autocrine and paracrine manner. It enhances the granulosa cell proliferation, aromatase activity, and progesterone biosynthesis (Gong et al. 1994; Rao et al. 2011). IGF-1 also plays an important role in the selection of preovulatory follicles by amplifying the actions of FSH/LH (Monget et al. 1993).

In the sheep, IGF-I primarily stimulates the proliferation of granulosa cells from small (1–3 mm diameter) but not from large (>5 mm in diameter) follicles. In contrast, IGF-I stimulates the secretion of progesterone by granulosa cells from large but not small follicles. Thus, IGF-I stimulates either proliferation or differentiation of granulosa cells, depending on the stage of development of follicles (Gong et al., 1996; Monniaux et al. 1997). In lactating cows, due to metabolic stress, decreased IGF-1 concentration and reduced expression of pregnancy-associated plasma protein-A (PAPP-A) in the granulose cells in the dominant follicle were reported as compared to nonlactating animals (Sanchez et al. 2014). Exposure of oocytes to a high concentration of IGF-I for a short duration increased inner cell mass proliferation during in vitro blastocyst formation in bovine (Velazquez et al. 2012).

The growth factors may also play an important regulatory role in antral follicular developmental processes. The only competent mature follicles in a wave will be able to develop in the presence of decreasing serum concentration of FSH. The IGF family is likely to be important in the selection of dominant follicles (Rao et al. 2011). The IGF-I and estradiol potentiate the action of FSH on granulosa cells differentiation. The increase in bioavailable IGF in large antral follicles during terminal follicular development (Gong and Webb 1996) and high responsiveness of granulosa cells to FSH during final follicular growth results from intrafollicular synthesis.

In vitro supplementation of LH, IGF-1, and EGF increased the expression level of vascular endothelial growth factor in luteal cell and granulosa cells culture of the preovulatory follicle. These growth factors play an important role in stimulating luteal and preovulatory follicular angiogenesis in buffalo corpus luteum (Chouhan et al. 2015) and follicles (Babitha et al. 2014), respectively.

From the recent findings, it is evident that growth factors are likely determinant for the development of small follicles before they become gonadotrophin dependent, but whether they play an essential role in the development of gonadotrophin-dependent follicles remains to be determined.

5.3   Gonadotrophins Secretion

Insulin and glucose metabolism are involved intimately with pituitary-ovarian function in ruminants (Downing et al. 1995a, 1995b). Insulin could affect GnRH or LH secretion by affecting the level of glucose or amino acid metabolism (McCann and Hansel 1986; Downing et al. 1995a, b). This hormone may alter the activity of the follicular cells either alone or in synergy with gonadotrophins (Gong et al. 1994).

A study from sheep revealed that insulin infusion during the luteal phase of the estrous cycle delayed preovulatory decline in FSH concentration by 8 h, and this could be due to an indirect effect associated with reduced secretion of estradiol and inhibin during the follicular phase (Downing and Scaramuzzi 1997). In sows, insulin treatment increased FSH concentration only during the first 24 h after injection (Cox et al. 1987). Contrarily, administration of insulin @ 40 IU two times a day and 0.4 IU/kg body weight could not produce any detectable changes in the FSH secretion in cattle (Harrison and Randel 1986) and pigs (Matamoros et al. 1991), respectively.

Insulin has been reported to increase LH production in cultured pituitary cells (Adashi et al. 1981). In ewes, administration of insulin during the midluteal phase or follicular phase did not affect characteristic preovulatory LH surges (Beam and Holcombe 1992), indicating that mechanisms controlling the frequency of LH pulses in ruminants may not be sensitive to serum insulin concentration. In contrast, LH pulse frequency (4.3 ± 0.4 vs. 1.8 ± 0.3 pulses/day) and mean LH concentration (0.48 ± 0.04 vs. 0.32 ± 0.03 ng/ml) were significantly reduced by insulin infusion during midcycle and $PGF_2\alpha$ given at the end of infusion (Downing and Scaramuzzi 1997).

5.4   Steroidogenesis

Insulin and IGF-I either alone or in combination with gonadotrophins have been found to have a profound effect on steroidogenesis of cultured bovine granulosa and luteal cells (Gong et al. 1993b; Baithalu et al. 2013). The addition of insulin and IGF-I to the granulosa cell culture medium on cattle and pigs stimulated estradiol secretion (Gong et al. 1994; Gutierrez et al. 1997) in a dose-dependent manner. Similarly, the administration of insulin increased the concentration of estradiol levels in the follicular fluid in heifers (Simpson et al. 1994) and ovulation rate in sheep (Downing and Scaramuzzi 1997). The reduced protein and mRNA expression of insulin receptor and alpha p85 of PI3K alter insulin signaling pathway (Hein et al. 2015) that could affect normal follicular steroidogenesis. The level of IGF-I was less

in follicular fluid of cystic cows, suggesting that the animals with cystic ovary had an altered regulation of the IGF system in the bovine ovary (Rodríguez et al. 2015).

The IGF-I and/or insulin may have a direct effect on the follicles and corpus luteum, resulting in the increased peripheral concentration of progesterone (Lucy et al. 1993). Insulin increases aromatase activity (Dorrington et al. 1987) and progesterone production in the porcine granulosa (May and Schomberg 1981) and bovine luteal (Sauerwein et al. 1992) cells culture. The higher release of progesterone by insulin was observed during the late (15–18d) luteal phase cell culture (Sauerwein et al. 1992). Administration of insulin for 4 days in sheep during the follicular phase decreases serum progesterone levels during the treatment period, suggesting the influence of insulin on corpus luteum regression or steroidogenesis (Beam and Holcombe 1992). In heifers, progesterone concentration (ng/ml) in the follicular fluid has been reported lower (71 ± 24 vs. 178 ± 19) in insulin-treated than control cows (Simpson et al. 1994).

The type-I IGF receptors are present in the corpus luteum. IGF-I enhances FSH-stimulated estrogen and progesterone production in granulosa cells of bovine (Gong et al. 1994) and ovine (Monniaux and Pisselet 1992). In theca cells, IGF-I synergizes with LH, increases LH receptors that augment androgen biosynthesis (Nahum et al. 1995). In granulosa cells, IGF-I increases FSH-mediated estrogen synthesis (Adashi et al. 1985) and after luteinization in synergy with LH stimulates progesterone synthesis. In the corpus luteum, the IGF-I-binding site increases during the luteal phase and attains maximum toward the end of the luteal phase.

The effect of IGF-I and insulin on luteal secretion changes with the stage of the cycle. Though insulin and IGF-I could effectively stimulate progesterone secretion from the late luteal stages, the total release of progesterone is limited to IGF-I stimulation alone (Sauerwein et al. 1992). The rise in plasma IGF-I at estrus is regulated by follicular estrogen. The follicular estradiol at estrus increases uterine IGF-I synthesis, which acts in an endocrine manner via type-I IGF receptor to affect late follicular or early luteal development (Perks et al. 1995).

5.5 Oocyte Maturation and Embryo Quality

Insulin plays an important role in oocyte maturation. Insulin induces bovine cumulus cell expansion and has a positive effect on embryo development in vitro (Zhang et al. 1991). Similarly, IGF-I is also an important mediator of follicular development, oocyte maturation, and subsequent embryo development. Follicles containing mature oocytes that failed to fertilize in vitro had a lower concentration of IGF-I (Jimena et al. 1992). IGF-I has been found to mediate and amplify estradiol action, which is essential for oocyte maturation.

The peripheral concentration of metabolic hormones has been positively correlated with the number of transferable embryos in cattle (Gong et al. 1993a, 1996). Elevated IGF-I levels in oviductal secretion have been observed at the time of embryo passage in cyclic cows (Herrler et al. 1992). The oviductal cells and preimplantation buffalo embryo possess IGF-I and insulin receptors (Daliri et al.

1999). Supplementation of culture media with insulin and IGF-I stimulates early embryonic development (Totey et al. 1996). Further, IGF-I reduced the deleterious effect of heat shock by slightly improving in vitro oocytes nuclear maturation and cleavage rates following in vitro fertilization in bovines (Meiyu et al. 2014). This embryonic development is supported by both autocrine and paracrine actions of insulin and IGF-I. A beneficial effect of insulin was observed on folliculogenesis and steroidogenesis in superovulated goats (Selvaraju et al. 2003). IGF-1 treatment improves the oocyte developmental competence by regulating the PI3K/Akt and apoptosis signaling (Javvaji et al. 2020).

5.6 Estrous Response

The onset of estrus was not affected in ewes treated with insulin after MAP sponge removal (Kirkwood et al. 1991). The estrous cycle length did not differ significantly in insulin-treated (during the luteal phase) ewes as compared to control (Beam and Holcombe 1992). Though earlier studies in pigs reported no effect of insulin on the weaning to estrous interval (Ramirez et al., 1997), later study reported a decrease in weaning to the estrus interval in pigs treated with insulin after weaning and opined that exogenous insulin creates a general anabolic signal that resulted in shortening the weaning to the estrus interval (Whitley et al. 1998).

5.7 Ovulation Rate and Superovulatory Response

The superovulatory response is positively correlated with the number of small follicles present in the ovaries at the beginning of FSH injection, and attempts were made to increase the number of small (2–5 mm diameter) antral follicles in cattle (Gong et al. 1993b) and goat through growth hormones and insulin (Selvaraju et al. 2002). Priming with rbGH in FSH-treated heifers increased ovulation rate and recovery of embryos. Treatment with rbGH stimulates follicular development and maturation through the increase in the peripheral concentration of insulin and IGF-I (Gong et al. 1993b). Further, increased follicular and peripheral IGF-I levels have been reported after insulin treatment in cattle (Simpson et al. 1994), ewes (Kirkwood et al. 1991) and sows (Cox et al. 1987).

Low fertilization rate and recovery of abnormal embryos continue to be the major limiting factors following superovulation and embryo collection in farm animals. This has been attributed to aberrations in oocyte maturation and asynchrony between maturational events between oocyte and follicle. Ovarian hyperstimulation and abnormal steroid levels are not only harmful to the egg quality but also to the uterine environment affecting the fertilization and embryo development. The circulating concentration of insulin and IGF-I has been associated with increased ovulation rate in cattle (Gong et al. 1993b) without alteration in serum LH profiles (Harrison and Randel 1986). Interaction of insulin and IGF-I with FSH might be physiologically important to increase ovulation rate due to the effect of these metabolic hormones in

decreasing follicular atresia (Cox et al. 1987). The rise in plasma insulin mobilizes more glucose supply to the follicles, which enhances ovulation rate without significant change in gonadotrophin concentration in ewes (Downing et al. 1995b). Insulin and glucose infusions inhibit the secretion of oestradiol in ewes. The suppressive effect of insulin was suggested to allow the selection of more than one ovulatory follicle leading to multiple ovulation (Downing et al. 1995b).

5.8   Metabolic Hormones on Improving Conception Rate

Reproductive wastage due to repeat breeding is a common reproductive problem in dairy cattle. The incidence of infertility in India ranges from 15 to 33.85 percent (Khan et al. 2016; Dutta et al. 2019), the majority of which is due to repeat breeding in cattle (Thakor and Patel 2013). The causes of repeat breeding have been attributed to genetic abnormalities, senility, nutritional deficiency, periestrus hormonal asynchrony, delayed ovulation, inadequate luteal function, and managemental factors (Maurer and Echternkamp 1982). These causes lead to either fertilization failure or early embryonic mortality. The repeat breeding condition due to fertilization failure/early embryonic mortality might originate from poor egg formation either during the early stages of follicle maturation and/or in the immediate preovulatory stages of follicular development. Repeat breeding animals had a significantly lower number of 1 to 3 mm size follicles. However, no difference was observed in 4 to 7 mm and > 8 mm diameter of follicles, corpus luteum weight, and the number of corpora albicantia (Maurer and Echternkamp 1985). It was concluded that repeat breeding females possess a smaller population of antral follicles or an endocrine status insufficient for oogenesis. In the repeat breeding cows, the disturbances in endocrine profile at periestrus leading to poor egg quality (Kurykin et al. 2011).

Early embryonic mortality in cattle has also been due to the inadequate functioning of corpus luteum. The delayed formation of corpus luteum with or without lowered secretion of progesterone during the luteal phase leads to luteal dysfunction. The possible causes of luteal dysfunction are abnormal folliculogenesis, inadequate or inappropriate timing of preovulatory LH surge, hormonal asynchrony at periestrus period, delayed ovulation, and lack of LH support during the luteal phase. The ability of a mature oocyte to fertilize and develop into competent embryo depends on the changes in its follicular microenvironment before ovulation. The higher progesterone and lower levels of LH at estrus could affect normal embryo development. An altered follicular milieu by the hormonal asynchrony during periestrus period also causes resumption of meiosis before the LH surge, leading to ovulation of an excessively aged oocyte.

Since metabolic hormones regulate growth, maturation, and ovulation of follicular and also luteal function, the modulation of metabolic hormones may regulate fertility in infertile animals. Though the half-life of insulin in ruminants is approximately 12 to 13 minutes (Trenkle 1972), modified insulin preparations with longer biological half-life are available and can be used to improve fertility in animals

(Selvaraju et al. 2002; 2012). The administration of long-acting insulin at concentrations of 1.0 IU/kg bodyweight for 3 days given at 6, 28, and 50 h after 60 mg MAP sponge removal did not affect the pregnancy rate (69 vs. 74%) and subsequent litter size (1.65 vs. 1.69) as compared to control in ewes (Kirkwood et al. 1991). In another study, in ewes, insulin administration at concentrations of 1.0 IU/kg body weight from day 12 of the estrous cycle to 24 h after estrus did not increase pregnancy rate (63 vs. 80%) and subsequent litter size (2.2 ± 0.3 vs. 1.9 ± 0.3) as compared to control (Beam and Holcombe 1992). Contrarily, sows receiving insulin injection 0.4 IU/kg body weight once daily for 4 consecutive days beginning on the day of weaning increases farrowing rate (92.3 vs 76.7%) as compared to control. It was opined that apart from the action of insulin on ovarian function, it also affects the establishment of pregnancy (Ramierz et al. 1997). However, with the same treatment schedule, no effect on pregnancy rate (85.7 ± 3.6 vs. $88.8 \pm 3.2\%$) and embryo survival (53.0 ± 5.70 vs. 51.0 ± 5.40) over the control in sows were also reported (Whitley et al. 1998). The beneficial effect of insulin on fertility in repeat breeder cattle has been observed (Selvaraju et al., 2002b).

Ovarian steroids (estradiol and progesterone) and protein hormones especially GH influence insulin secretion (McCann and Reimers 1985). Basal insulin concentration was greater at estrus than at diestrus and nonlactating pregnant cows had lower serum insulin concentration than the nonlactating nonpregnant cows. However, progesterone increases the ability of the beta-cells to secrete insulin in response to intravenous administration of glucose in the rat (Ashby et al. 1978). Similar way, estradiol treatment produces hypoinsulinemia in cows (Laarveld et al. 1982). However, it has not been established how exogenous steroids affect insulin secretion. The level of milk yield was negatively associated with plasma insulin and IGF-I concentration. But the concentration of IGF-I in milk was not associated with fertility (Taylor et al. 2004). The growth hormone treatment stimulates follicular development, the advancement of puberty in heifers (Cooke et al. 2013), and the conception rate in cattle (Ribeiro et al. 2013), but not embryo production in buffalo (Ferraz et al. 2015).

5.9 Effect of Metabolic Hormones and Male Fertility

Impaired male fertility is a major problem in animal breeding and may have a significant effect on the breeding population, because the ratio of males to females is extended particularly when AI is used. Glucose, amino acids, or nutrient-related metabolites, such as insulin, growth hormone, and the insulin-like growth factors, influence the hypothalamo-hypophyseal-gonadal axis for supporting gametogenesis and steroidogenesis. These nutritional effects could continue to affect pre- and post-fertilization events within the female reproductive tract. The biochemical mechanisms underlying sperm maturation and the development of motility and fertilizing ability remain largely undefined. The ability of spermatozoa to travel and fertilize the egg develops during their transit through the epididymis; this process is likely controlled by seminal plasma composition.

The presence of various growth factors, IGFs, EGF, and TGFβ and unidentified growth factors in seminal plasma plays a regulatory role in sperm physiology or reproductive tract maintenance (Patil et al. 2020). IGF-I has been proposed to have a direct or indirect role in spermatogenesis/steroidogenesis in the testis and its derangement may be involved in male infertility. Recently, considerable research has been carried out on the relationships between IGF-I and male reproductive traits in breeding animals. The spermatogonial stem cells development is influenced by the growth factors including IGF-1 (Binsila et al. 2020). IGF-I is a mitotic polypeptide that stimulates glucose uptake by various cells. Its effects on female reproductive functions have been extensively studied. However, there has been little research on relationships between IGF-I and male reproductive traits in breeding animals.

IGF-I in seminal plasma was shown to be primarily of testicular or epididymal origin. In several species, IGF-I and IGF-II are regulators of testicular and also be post-testicular regulators of reproductive function. Scrotal circumference and percentage of normal sperm cells are related to blood serum IGF-I concentration in yearling Angus bulls (Yilmaz et al. 2004). When the rams are supplemented with nonconventional protein rich source such as detoxified Karanjia cake in the diet at above a certain level affects the expression of IGF1 receptors in the testis and thus the semen production capacity (Dineshkumar et al. 2013).

IGF-I maintains sperm motility in vitro either through energy metabolism or its antioxidant effect (Henricks et al. 1998). The effects of IGF-I on capacitation have not yet been evaluated. Since the IGF-I cell surface receptor has the tyrosine kinase domain, and the tyrosine phosphorylation has a key role in capacitation/acrosome exocytosis, IGF-I could also potentially modulate the mature sperm cell function. It was also suggested that IGF-I may affect sperm motion characteristics that are typical of hyperactivated sperm (Henricks et al. 1998). These sperm-motion characteristics are important in penetration of the zona pellucida and transport through oviductal mucus. The IGF-I may influence the semen quality by acting as antioxidants and protecting sperm membrane quality (Selvaraju et al. 2009; Susilowati et al. 2015). A study from buffalo also suggests that addition of IGF-I in the semen extender improves the cryosurvivability of sperm (Selvaraju et al. 2016; Kumar et al. 2019).

Modulating IGF I concentration in seminal plasma of healthy breeding bulls by diet may improve their fertilization efficiency. The modulation of components of the IGF system in the testis in healthy breeding bulls increases their fertilization efficiency (Sauerwein et al. 2000), but a study from this lab revealed that the IGF-I improved sperm function and not in vitro fertility (Selvaraju et al. 2010). In mature rams, peripheral concentrations of IGF-I are affected by dietary composition mainly through changing energy levels either by carbohydrate rich grain source (Selvaraju et al. 2012a) or by oil rich in polyunsaturated fatty acids (Selvaraju et al. 2012b). Plant oils with predominantly polyunsaturated fatty acids have also been shown to increase serum and seminal plasma concentrations of IGF-I rams (Selvaraju et al., 2012). Since IGF-I is a testicular and post-testicular regulator of reproductive function, modulating the serum and seminal plasma IGF I level may improve semen quality and fertility.

Studies indicate that modulation of metabolic hormones levels in the testis may influence spermatogenesis and steroidogenesis and such regulation is highly important in the cattle industry to improve fertility. Through dietary energy, seminal plasma IGF-I levels can be modulated and the optimum level of seminal plasma IGF-I required for maintaining optimum semen quality needs to be established.

5.10 Conclusion

The metabolic hormones reflect the nutritional status and influence the reproductive function in males and females. These regulators are responsible for maintaining functional competence of gametes and fertility. Dietary modulation of the metabolic hormones may improve the reproductive efficiency in livestock.

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Salivary Crystallization and miRNA: Potential Biomarkers for Estrus Identification in Buffaloes

Varun Kumar, Ravinder, Prashant Singh, Gangu Naidu Surla, Dheer Singh, and Suneel Kumar Onteru

Abstract

Estrus detection and properly timed insemination in buffaloes remains as a challenge, especially under smallholder production systems, wherein application of automation and sensor-based tools for reproduction management is not possible. Traditionally, manual observation of animals for the signs of estrus detection is being practiced in developing countries, which is labor intensive and often results in reduced estrus detection efficiency and accuracy, as a majority of the buffalo shows estrus onset/behavior during night or early morning hours. Further, not all buffaloes show overt estrus signs so that they can be visually observed. The incidence of silent estrus is very high in buffaloes, and, estrus detection is really a challenge in this species especially during low-breeding season. In the recent past, several attempts have been made to identify biomarkers in body fluids and to develop cow-side test for estrus detection. In this chapter, the use of salivary crystallization patterns and salivary molecules as an aid for estrus detection is detailed.

Keywords

Estrus detection · Salivary fern pattern · Buffaloes · miRNAs · Fertility

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6.1 Introduction

Estrus detection needs further efficient methods for buffaloes compared to other domestic animals because of their short estrus duration (5–27 h) and absence of clear estrus behavior. Statistically, only 50% of the estrus or heat symptoms in buffaloes are detected with 41% estrus detection efficiency. This leads to the improper insemination timings in both the organized farms (20.75%) and field (30%) conditions (Srivastava et al. 2013). In India alone, around 13.62 million buffaloes (25%) out of 54.48 million buffaloes are improperly detected for estrus symptoms. A similar scenario can also be expected in other developing countries. One missing estrus in buffaloes causes an estimated loss of INR. 5901 to 7728 to the buffalo farmers (Abdullah et al. 2014), which subsequently leads to nearly INR 45000 million loss to the Indian economy alone. Therefore, efficient and easy estrus identification is essential for buffalo farmers. There are many different methods available for detection of heat in domestic livestock, such as chalk tail head (Macmillan and Curnow 1977), continuous videotaping (Donaldson et al. 1968), visual observation (Williamson et al. 1972), pressure sensation device (Shipka 2000), pedometers (Arney et al. 1994), and many more. Among them, pedometers and plasma progesterone detection (Delwiche et al. 2001) appear to have 100% efficiency in heat detection. However, majority of all these heat-detecting methods are not studied properly in buffaloes. Moreover, these methods require skilled personnel, which is impractical in rural countries like India. Hence, there is an urgent need of accurate, most efficient, reliable, and field applicable heat-detecting method for buffaloes.

To overcome the heat or estrus detection problem in buffaloes, researchers are trying to explore biomarkers in noninvasive fluids. The noninvasive fluids include milk, urine, cervico-vaginal fluid, feces, and saliva, which are the greater resources of biomarkers. Among these fluids, saliva is available every time irrespective of any physiological stage. In addition, its collection is cost effective (Yoshizawa et al. 2013), and it appears to have long period of stability (Ang et al. 2011). Therefore, the saliva is one of the ideal biofluids for the discovery of biomarkers for estrus in buffaloes. Particularly, the cell-free saliva is better to represent the physiological status of animals as it is a combination of partial ultrafiltrate of the plasma as well as the secretion of salivary glands.

The biomarkers might be mi-RNA/small-RNA, carbohydrates, proteins, lipids, steroids, nucleic acids, ions, and physiochemical/morphological characteristics like crystallization pattern formations of body fluids. Any of these biomolecules and morphological characteristics specifically associated with estrus can be served as biomarkers for estrus detection. In the recent times, the detection of miRNA in major body fluids gained importance, since it reflects the various physiological conditions of the animal. In addition, the sensitivity and specificity of RNA-based biomarkers are better than protein biomarkers. Further, the cost of RNA biomarker detection is cheaper than protein biomarker detection as RNA-based biomarker can be detected by field applicable RT-LAMP (Reverse-transcriptase loop-mediated isothermal

amplification) technology without the synthesis of antibodies, which are needed for a protein biomarker (Xi et al. 2017). On the other hand, physical patterns like ferning/crystallization pattern of the biofluids serve as a major indicator of animal reproductive status. Therefore, by quantifying the salivary miRNA and characterization of fern patterns, it is possible to develop an estrus identification method in buffaloes.

6.2   Salivary Crystallization

Crystallization of body fluid was first described by Papanicolaou (1946) in vagino-cervical mucus of women. Garm and Skjerven (1952) noticed the cervico-vaginal crystallization of cows and reported that during estrus, the fern morphology crystals appeared, and they disappeared during the luteal phase of the estrous cycle. Crystallization was also described in nasal mucus (Peterson 1984), tears (Golding and Brennan 1989), milk or colostrum (Zondek and Rozin 1954), and saliva (Pardo-Carmona et al. 2010). Among these fluids, saliva crystallization is easy to perform. In the recent years, monitoring of menstrual cycle in women was carried out by observing salivary fern patterns. To observe the salivary fern patterns by women themselves, different kinds of small hand-held microscopes or pocket microscopes or paper microscopes are currently available in the market. These pocket microscopes are giving an average accuracy of 92% (Guida et al. 1993). In the recent years, the salivary crystallization method is also used to determine the ideal mating time in bitches (Pardo-Carmona et al. 2010), monitoring of menstrual cycle in wild female Bornean orangutan (Kubatova and Fedorova 2016) and in cattle for determining early pregnancy diagnosis after AI (Skalova et al. 2013) as well as for determining estrus time (Gnanamuthu and Rameshkumar 2015). The researchers hypothesized that estrogen has positive influence on salivary crystallization. However, the exact mechanism for the appearance of typical fern pattern is not yet clear.

The salivary crystallization method was also used for the first time to determine the estrus stages in buffaloes in our lab (Ravinder et al. 2016). The salivary fern patterns were observed throughout the estrus cycle of buffaloes, and fern patterns were also quantified by fractal analysis method. In this study, a total of 450 saliva samples were collected from 8 female nonpregnant Murrah buffaloes for two purposes: First, to prepare a smear on glass slides by using a standard blood smear technique for observing different types of saliva crystallization or ferning patterns; second, to estimate the salivary estradiol and progesterone concentrations. The smears showed different crystallization patterns throughout the estrus cycle, and they are categorized into typical symmetrical fern-like, branch-like, fir-like, combination of fir-fern branch, dotted, and nontype (Figs. 6.1 and 6.2). The typical symmetrical fern-like pattern appeared during the estrus stage with a proportion of 0.84 ($P < 0.01$), which was a higher proportion than the general proportion of estrus detection (0.50/50%) in buffaloes in the field condition. The salivary levels of total estradiol and E2/P4 ratio were found to be higher ($P < 0.05$) at the estrus stage than
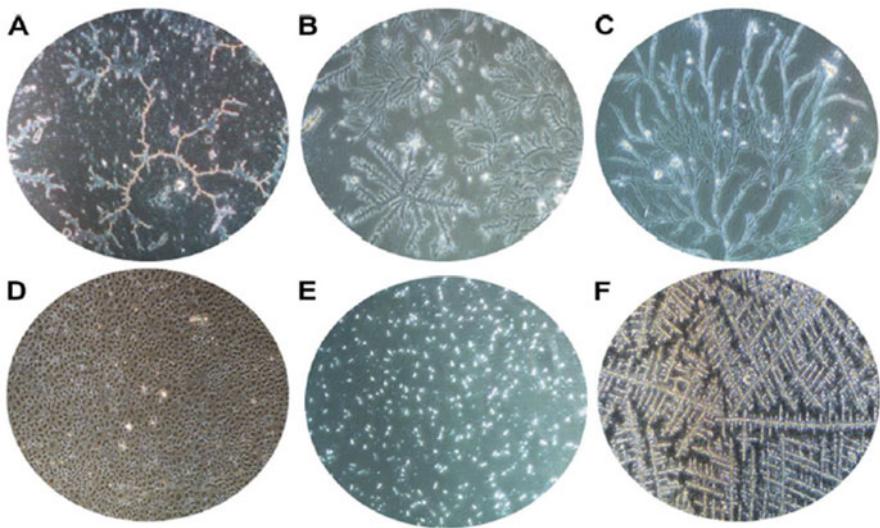
Fig. 6.1 Different salivary crystallization patterns in buffaloes. Branch-like (**a**), fern-like (**b**), fir-like (**c**), none (**d**), dotted (**e**), and typical fern patterns (**f**). (This image was taken from Ravinder et al. 2016 for educational purpose)
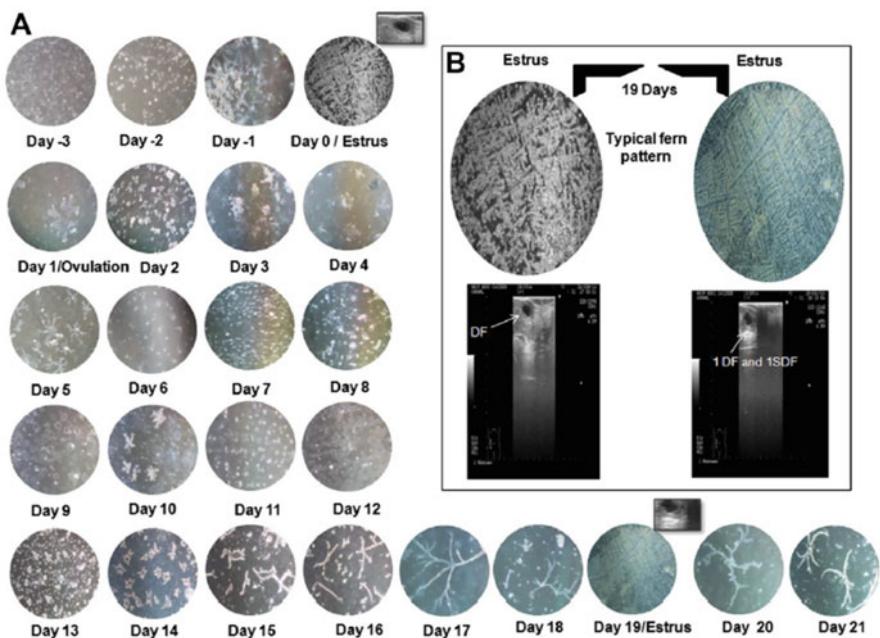
Fig. 6.2 Salivary fern patterns during estrus cycle of buffaloes along with the ultrasound images of ovaries. (This image was taken from Ravinder et al. 2016 for education purpose)
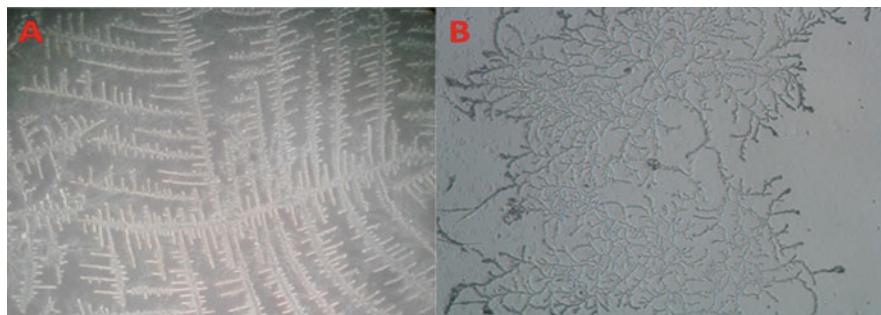
Fig. 6.3 (**a**) Estrus and (**b**) Diestrous stages. The day 10 of the estrous cycle was considered as the diestrous stage. (This image was taken from Surla et al. 2021 of for educational purpose)

the diestrus stage, and the salivary progesterone levels were significantly higher at the diestrus stage than the estrus stage (Fig. 6.3). On the basis of the above findings, it can be concluded that the higher levels of estradiol during estrus stage cause the typical fern-like crystallization patterns of the saliva in buffaloes and it can be applied in the field condition for determining the estrus in buffaloes.

The saliva crystallization pattern method was later validated in more than 500 estrus events (Surla et al. 2021). In this study, 4 buffaloes population samples were considered: organized herd (PS1), buffaloes came for AI (PS2), buffaloes with induced estrus by PGF2α (PS3), and random buffaloes at farmers doorsteps (PS4). In the PS1, ten buffaloes were exclusively studied for this research. From this PS1, saliva samples were collected from 149 potential estrus stages over a period of one year, in which 111 samples showed typical fern-like patterns before 8–12 h of the actual estrus behavioral sings. In the PS2, a total of 114 buffaloes were observed for salivary fern patterns, which were brought for AI centers. Among them 44 buffaloes confirmed for typical fern patterns in which 15 buffaloes were found to be pregnant after AI and 38 buffaloes did not show any typical fern patterns, but still 24 buffaloes became pregnant after AI. In the PS3, 44 buffaloes were induced for estrus by administering a single dose of PGF2α in which only 7 buffaloes were found to show typical fern-like crystallization patterns. In the last population sample, PS4, a total of 275 buffaloes with unknown reproductive history and absence of estrus sings were considered and the typical fern-like crystallization patterns were observed by using a paper-based microscope called Foldscope (Fig. 6.4). Among them, only 22 buffaloes have shown typical fern patterns, and 20 of the buffaloes were confirmed for estrus by veterinarians. These data and observations, and a significant higher proportion of estrus identification in the PS1 and PS4 on the basis of salivary fern-like patterns, further support the utility of saliva crystallization-based estrus identification in the field conditions: farmers are trained for the detection of saliva crystallization patterns by using a simple filed applicable microscope.
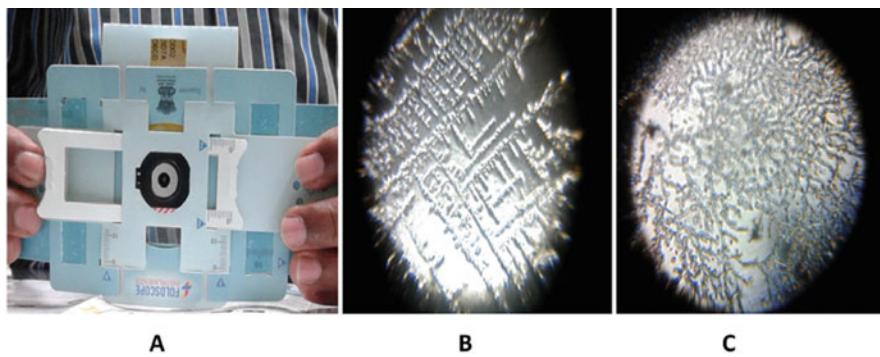
Fig. 6.4 Salivary fern patterns of buffaloes by the Foldscope. (**a**) Foldscope. (**b**) Estrus stage (**c**) Diestrous stages. The day 10 of the estrous cycle was considered as the diestrous stage (This image is taken from the journal reproductive biology for educational purpose)

6.3   Chemical Composition of the Saliva Fern Patterns

The mucus/tear/saliva forms fern-leaf-like patterns when they are allowed to dry on a clean and smooth surface, like a glass slide. The mechanism behind the ferning is still a little mystery for researchers. Ferning patterns in tears were extensively studied in humans to address various eye problems. Many hypotheses have been put forward to explain the ferning or crystallization. Golding and Brenan (1989) hypothesized that ferning is not dependent on any single chemical species but the interrelationship of many different chemical species. Vaikoussis et al. (1994) suggested that the balance between sodium chloride and mucus is important for ferning. According to Kogbe et al. (1991), for successful ferning, the ratio between divalent calcium and magnesium ions to monovalent sodium and potassium ions plays a major role. Pearce and Tomlison (2000) studied the chemical composition of human tear ferns and analyzed the locations of chemical elements in the fern. When tear fern patterns were examined under a scanning electron microscope, two main structures were observed: dendrites and cubical crystals. Dendrites make up a major part of the fern, whereas cubical crystals are present adjacent to the dendrites. X-ray analysis of the dendrites and cubical crystals structures showed that the dendrites are made up of NaCl, along with other ions, and cubical crystals are made up of KCl. In this study, sulfur was also detected at the peripheral region of the tear smears, which suggests the possibility of presence of macromolecules like mucins and proteins at the periphery of the smears. Further, it was inferred that these macromolecules deposited at the very periphery of the dry teardrop may play a role in retardation of fern growth (Golding et al. 1994). These results propose that the macromolecules such as glycoproteins proteins and mucins may have some role in fern formation. This was the first study regarding the spatial location of organic (sulfur-containing) molecules in the fern. Such studies on buffalo saliva fern patterns are underway.

6.4   Salivary Mucins/Glycoproteins and Electrolytes

The appearance of typical fern-like patterns at the estrus stage is mainly influenced by increased estrogen levels. However, the exact mechanism for the appearance of typical fern pattern is not known. One of the causes for typical fern-like crystallization patterns of saliva could be salivary mucins and their associated carbohydrates and salts. Mucins are high-molecular-weight proteins composed of protein core and carbohydrate side chain (50–80% CHO glyco-conjugated). In human saliva, higher-molecular-weight mucins account for about 30% of total salivary mucins containing 12–15% of protein, 80–85% carbohydrates like sialic acid, fucose, etc. It is clear that mucins are majorly made up of large protein polypeptides and oligosaccharides side chains (Wu et al. 1994). However, specific mucins and their related carbohydrates and salts associated with typical salivary fern patterns at the estrus stage of buffaloes were not yet delineated. Identifying these molecules will help in developing a saliva-based color reaction for estrus identification in buffaloes.

The effect of hormones like estrogen and progesterone on electrolyte levels was well studied in humans. The concentrations of sodium, potassium, and chloride ions were observed to increase in parallel with the estrogen level during the ovulatory phase. The salivary ferning is formed by NaCl, which rises under the effect of estrogen (Alagendran et al. 2013) and decreases with progesterone (Macdonald 1969; Linford 1974). This observation proves that these salts along with hormones help in fern forming and brings out the day of fertile period in women (Pattanasuttinont et al. 2007). A study conducted by Dadlani et al. (1982) found that the same kind of changes occurs in the concentration of electrolytes corresponding to hormonal levels in serum during the menstrual cycle. It can be understood that the ovarian hormones have a good connection with electrolytes, which can be used for the prediction of ovulation as a noninvasive method.

Devi et al. (2016) quantified electrolytes concentration in saliva of Murrah buffaloes during estrus stage. They found that the concentration of calcium (8.76 $\pm$ 0.08–12.11 $\pm$ 0.11 mg/dl), inorganic phosphorus (6.56 $\pm$ 0.13–14.72 $\pm$ 4.50 mg/dl), magnesium (2.27 $\pm$ 0.14–5.79 $\pm$ 0.15 mg/dl), sodium (139.47 $\pm$ 0.31–159.62 $\pm$ 1.22 mmol/L), potassium (12.40 $\pm$ 0.22–-26.85 $\pm$ 1.22 mmol/L), and chloride (109.28 $\pm$ 0.41–137.07 $\pm$ 0.68 mmol/L) varied during the different phases of estrous cycle. However, during the estrus phase of the cycle, the concentration of all electrolytes was found to be significantly much higher compared to other phases of estrous cycle. This study found that the concentration of salivary electrolytes was positively associated with estrogen concentration and negatively associated with progesterone level.

6.5   Salivary miRNA

MicroRNAs are small (19–22 nucleotides), single-stranded RNA molecules, which regulate various biological process. They are synthesized in the nucleus of the cells in the presence of enzymes Drosha (RNA III endonuclease) and other

microprocessor complex proteins like DGCR8. The newly synthesized pre-miRNAs are then transported to cytoplasm by exprotein-5 and Ran-GTP. In the cytoplasm, pre-RNA is converted to mature miRNA by a Dicer complex. The mature miRNA participates in posttranscriptional regulation of gene expression either by repressing the translation or degradation of targeted mRNA molecules. In addition to intracellular function of miRNA within the cells from which they originate, they are secreted out of the cells through nanosecretory vesicles called exosomes.

miRNAs are omnipotent in all types of body fluids like serum, plasma, saliva, tear, and urine. When compared to other biomolecules like proteins and other RNAs, miRNAs are more stable in harsh conditions like low or high pH and they can be stored for long term. This property is because of their encapsulation within the lipoprotein complex called microvesicles or exosomes. Additionally, they are easy to detect using synthetic complementary oligonucleotides/PCR/DNA amplification. A large number of unique miRNAs like miR-182, miR450b-5p, miR-622, and many more have been identified in the saliva. Because of their stability, easy detection and most importantly their correlation with the different physio-pathological conditions, miRNAs can be used as biomarkers to monitor the health and reproductive physiology of farm animals.

Studies on salivary RNA-based biomarkers for estrus identification are very scanty in dairy animals. A study on buffaloes showed a suggestive higher abundance of heat shock protein 70 (*HSP70*) and toll-like receptor 4 (*TLR4*) transcripts in the buffalo saliva at the estrus stage than diestrus stage (Onteru et al. 2016). The study emphasized the usage of the direct saliva transcript analysis without RNA isolation. Such a technique along with the integration of RT-LAMP technology would be useful to be applicable for using RNA biomarkers in the field conditions for buffaloes. Toward this goal, a recent study (Singh et al. 2017) on the investigation of estrogen responsive miRNAs (*miR-24, miR-200c, miR-191, miR-16 and miR-223*) as estrus biomarkers in buffaloes' saliva indicated that the miR-16, miR-191, and miR-223 showed their higher abundance in the buffalo saliva on the day 6 and 18–19 than that of the estrus and tenth day (diestrus) of estrous cycle. This observation indicated that the lower presence of these miRNA in buffalo saliva on the estrus and tenth day of estrous cycle could intuitively indicate the presence of dominant follicle on the ovary. It should be noted that the lower expression of these miRNA in the ovarian follicles is essential to upregulate certain biochemical pathways, such as fatty acid biosynthesis and oocyte meiosis, and certain target genes like *FGF, BDNF*, *IGF1, KRAS, BCL2,* and *IGF1*, which are well known to be involved in the development of a dominant follicle on the ovary. Taken together, these clues further reinforce the utility of saliva RNA as biomarkers for ovarian physiology, thus the estrus stage in buffaloes. On the basis of these observations, further omics studies such as salivary transcriptome and miRNome are needed to identify the miRNA biomarker specific to the estrus stage in buffaloes. One of the bottlenecks for such omics studies is the high-quality RNA isolation itself. On the basis of our experience, the primary requisite to perform transcriptome studies from buffalo cell-free saliva is to develop an efficient RNA isolation method. If such an RNA isolation method is

developed, more reliable miRNA biomarkers can be identified from buffalo saliva for estrus detection.

6.6   Conclusion

Saliva is the one of the easily available noninvasive fluid every time to explore the biomarkers for estrus identification in buffaloes. Among several prospective biomarkers, observation of typical fern-like crystallization patterns of the dried salivary smear is a simple and potential estrus identification method in buffaloes. However, other potential biomarkers could be salivary miRNA specific to the estrus stage, but their search is still at a preliminary stage. The exploration of salivary miRNA specific to the estrus stage by transcriptome studies would be fruitful if any good method is devised for the isolation of high-quality RNA from the cell-free saliva of buffaloes.

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Endometrial Toll-Like Receptors During the Reproductive Cycle and Uterine Infection in the Cow and Buffalo

7

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Abstract

Toll-like receptors (TLRs), which come under the class of pattern recognition receptors, mediate innate immune responses upon binding to the pathogen-associated molecular patterns (PAMPs). TLRs are present in a wide variety of tissues including the endometrium. Expression and regulation of endometrial TLR during the early postpartum period is important in the clearance of uterine infections in the bovine. This chapter updates the available evidence on the expression pattern of TLR in the endometrium, regulation by phases of the estrous cycle, and their significance in the postpartum clinical endometritis and subclinical endometritis.

Keywords

Toll-like receptors · Endometrium · Uterine infection · Immunity · Bovine

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7.1   Introduction

A healthy uterine environment is an essential prerequisite for successful establishment of pregnancy. Diseases of the uterus, especially the endometrial layer, prolong the luteal function, resulting in longer calving interval (Sheldon et al. 2009). Establishment of an active infection of the uterus depends on the complex interaction among the endometrial mucosal immune response, pathogen load, and the role of virulence factors. Uterine infections are, always almost, ascending in nature, which implies that the cervix ought to be patent. Though ascending infections can occur at estrus, postpartum uterine infection is common in the cow and buffalo (Raman and Bawa 1977; Azawi 2008). The economic importance of clinical (CE) and subclinical endometritis (SE) in the PP cow has been investigated by many authors (LeBlanc et al. 2002; Gilbert et al. 2005; Williams et al. 2007). There is consensus that the endometritis and associated inflammatory responses adversely affect the fertility and animal welfare in the cow (Sheldon and Dobson 2004) and buffaloes (Azawi 2010).

In the first week of PP, around 80–90% cow and buffaloes develop uterine infection, which is physiological after calving (Williams et al. 2007; Sheldon et al. 2008). Though most animals eliminate the infection and resolve the physiological inflammation by day 21 PP, a proportion of normally calving and most of the abnormally calving cows develop endometritis. A better understanding of innate immunity, in particular, its molecular mechanisms, is highly desirable as it may help in devising strategies for controlling the uterine infections (Martinon et al. 2009). The innate immune arm of the endometrium responds to pathogens through components of the complement system, production of antimicrobial peptides like beta-defensins, immunoglobulins, acute phase proteins, and activation of pattern recognition receptors (PRRs) (Wira and Fahey 2004). Germ-line-encoded PRRs can bind to the conserved pathogen-associated molecular patterns (PAMPs) present on the microorganisms, (Takeuchi and Akira 2010) and initiate the intracellular signaling events that result in the production of cytokines and chemokines to clear the infection. The PRRs comprise four main classes of soluble and membrane-bound molecules that include toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs). Despite the fact that endometrium of animals and humans expresses different PRRs (King et al. 2003; Hirata et al. 2005), only TLRs are intensively investigated in the context of postpartum bovine uterine infection.

7.2   Structure of TLR

TLRs belong to the subfamily of leucine-rich repeat (LRR) proteins and come under type I integral membrane glycoproteins and has three major domains. The extracellular N-terminal domain is made of about 16–28 LRR, and each LRR is composed of 20–30 amino acids with the conserved motif (Kawai and Akira 2010). A total of 10 TLRs have been characterized in the cow and buffalo (McGuire et al. 2005; Vahanan et al. 2008). Though the functionality of the TLRs is not demonstrated, it is

presumed that bacterial PAMPs would activate the cognate receptors upon binding and would help in clearing the endometrial infection in the cow and buffalo.

Important PAMPs of Gram-positive bacteria include cell wall molecules such as peptidoglycan, glycolipids, lipopeptides, and lipoteichoic acid that induce heterodimerization of TLR2/TLR1 or TLR2/TLR6 when they interact with the host cells (Kovacs-simon et al. 2011). Tri-acetylated lipopeptides bind to TLR2/TLR1, whereas di-acetylated lipopeptides bind to TLR2/TLR6 (Jin et al. 2007). Single-stranded RNAs from different viruses activate TLR7 and TLR8 pathway to upregulate interferon-stimulated genes. Resiquimod, which is an imidazoquinoline-like molecule, is used to mimic the effects of single-stranded RNA and is tested for its use as adjuvant in poultry vaccines (Matoo et al. 2018). Double-stranded RNA (dsRNA) of viral origin serves as ligand for TLR3 and its analog, polyinosinedeoxycytidylic acid I: C (Poly I:C), has been tested for its role as vaccine adjuvants. In the context of bovine and bubaline endometritis, Gram-negative bacterial lipopolysaccharide (LPS) has been evaluated in the clearance of uterine infection.

7.3   TLR Signaling Pathway

The LRR domains of TLRs are involved directly in the recognition of various components of microbes. The cognate ligands recognized by different TLRs are summarized in Table 7.1. Remarkably, despite the conservation among LRR domains, different TLRs can recognize several structurally unrelated ligands. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2, and TLR4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. By contrast, TLR3, TLR7, and TLR9, which are involved in the recognition of nucleic-acid-like structures, are not expressed on the cell surface. For instance, TLR9 has recently been shown to be expressed in the endoplasmic reticulum and is recruited to

Table 7.1 Cellular location of TLRs and their cognate ligands

S. N.	TLRs	Cellular location	Ligands
1	TLR1	Plasma membrane	Triacylated lipopeptides
2	TLR2	Plasma membrane	Peptidoglycan, lipoteichoic acid
3	TLR3	Endosome	Ss RNA and ds DNA
4	TLR4	Plasma membrane	LPS, LTA, fibronectin, mannan (Candida)
5	TLR5	Plasma membrane	Flagellin
6	TLR6	Plasma membrane	Diacylated lipopeptides, fungal
7	TLR7	Endosome	ssRNA, imidazoquinolines
8	TLR8	Endosome	Ss RNA
9	TLR9	Endosome	Bacterial and viral unmethylated CpG motif

Reproduced from Ganesan et al. (2013).

endosomal/lysosomal compartments after stimulation with CpG motif (Akira et al. 2006).

Ligand recognition by TLRs leads to the recruitment of various TIR-domain-containing adaptors such as myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein–inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). The engagement of TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR11 with their respective ligands recruits MyD88. In addition to MyD88, TLR1, TLR2, TLR4, and TLR6 recruit TIRAP, which serves as a linker adaptor between the TIR domain of TLRs and MyD88. Binding of ligand with TLR3 and TLR4 recruits TRIF. Furthermore, TLR4 recruits TRIF through TRAM, which links between TIR domain of TLR4 and TRIF. This recruitment of adaptors triggers the cascade of signaling pathway and ultimately the activation of transcription factors such as nuclear factor-κB (NFκB) and interferon regulatory factor (IRF) (Kawai and Akira 2010; Takeuchi and Akira 2010).

From clinical point of view, the cellular components of *E.coli* like LPS and flagellin will be recognized by TLR4 and 5, respectively, before it colonizes the endometrium. The cellular component of *A.pyogenes* such as triacylated lipoproteins and peptidoglycans are the potential ligands recognized by TLR2/TLR1 or TLR2/TLR6 (Kovacs-simon et al. 2011). TLR9 recognizes the unmethylated CpG motifs of single-stranded DNA present in the genomes of many viruses and bacteria, probably of IBR/IPVV of Herpes virus family, and *Brucella abortus* as they are rich in CpG motif.

7.4 Uterine Infection

Ascending infections of the bovine uterus occurs during the early postpartum and at estrus as the cervix is patent. Complex interaction among the bacterial load, virulence factors of the pathogen, and immunity of the cow determine whether an animal would clear the infection, or, the infection is established. Abnormal calving events such as uterine torsion, dystocia, and retention of fetal membranes predispose to increased bacterial overload (LeBlanc 2008). Sequential bacterial culture and metagenomic studies of uterine samples during the first 3 weeks PP indicate the dynamic change in the number of organisms; however, clinical or subclinical endometritis in the cow and buffalo is most often due to *Arcanobacterium pyogenes (formerly Corynebacterium pyogenes), Escherichia coli, Fusobacterium necrophorum, and Provetella spp* (Bonnett et al. 1991; Bicalho et al. 2010). *E.coli* isolated from the bovine uterus within day 10 PP expressed a battery of virulence factors: fimH gene has been shown to increase the risk of endometritis (Silva et al. 2009). Similar to enteropathogenic and uropathogenic *E. coli*, an endometrial pathogenic *E. coli* (EnPEC) has been shown to be adherent and invasive to endometrial cells and stimulated the production of inflammatory mediators like chemokines and PGE_2 in cell culture as well as mice model (Sheldon et al. 2010). Virulence factors associated with uterine *E coli* isolates are fimH, astA, cdt, kpsMII, ibeA, and

hlyA (Bicalho et al. 2010) of which fimH was common in the cows with metritis. In addition to *E. coli*, *A. pyogenes,* which contains the virulence factors such as pyolysin (plo) and a collagen-binding protein (CbpA), *causes* severe endometritis and infertility (Dohmen et al. 2000). It is believed that *E. coli* infection during the first week of PP predisposes the endometrium to subsequent infection with *A. pyogenes* (Olson et al. 1984; Williams et al. 2007). Subsequently, *A. pyogenes* acts synergistically with *F. necrophorum* and *Provetella sp.* to aggravate the uterine disease (Bonnett et al. 1991). *E. coli* releases bacterial cell-wall component lipopolysaccharide (Williams et al. 2008); *A. pyogenes* produces the cholesterol-dependent cytotoxin, pyolysin (Bicalho et al. 2012), and a growth factor for *F. necrophorum* (Sheldon and Dobson 2004); *F. necrophorum* produces a leukotoxin; and *Provetella sp* produces a substance that inhibits phagocytosis (Sheldon and Dobson 2004). Besides bacteria, bovine herpesvirus IV (BoHV-4) has been linked to cause uterine disease based on cell culture studies (Donofrio et al. 2007).

7.5   Endometrial TLR

In cattle, Davies et al. (2008) reported the expression of TLRs (TLR1 to 10) in the horn as well as body of uterus. Under in vitro conditions, immunolocalization of TLRs using immunohistochemistry and western blot studies would be essential to generate better information on the expression profile of endometrial toll-like receptors. Similarly, the ligand-receptor-based studies in the whole animal model would be essential to understand the functionality of the TLRs in terms of immune response generated following ligand-receptor binding.

The sex steroid hormones (estrogen and progesterone) influence the expression profile of various endometrial TLRs due to the functional dependency of endometrium to these hormones across the estrous cycle. Hence, to study the endometrial TLRs expression, ovariectomized animal would be the most suitable experimental model, as the hormonal milieu can be modulated by exogenous administration of estrogen and/or progesterone. Transcriptional profile of TLRs was also studied during different stages of pregnancy, and it was found that TLR4 and TLR5 showed highest expressions during third trimester of pregnancy in cattle. Conversely, TLR 1/6 tend to show increased transcription during first trimester of gestation in bovine (Silva et al. 2012). These data suggest that the uterus may be more responsive to pathogens during the period of pregnancy and subsequent parturition (Davies et al. 2008). An increased transcription of TLR 4 and TLR5 may be related to defense mechanism against such infections (King et al. 2003).

In the buffalo, Vahanan et al. (2008) reported the presence of TLR2, TLR5, TLR7, TLR8, TLR9, and TLR10 transcripts in the normal uterus of buffalo, irrespective of the influence of the different stages of estrous cycle. Unlike cow, buffalo lacks TLR1 expression in the endometrium (Ajevar et al. 2014: Kharayat et al. 2019). Quantitation of TLR4 transcripts in the bubaline endometrium during the follicular and luteal phase of estrous cycle and endometritis is reported, and it

was found that the expression of TLR4 was significantly higher both in luteal as well as follicular phase of clinical and subclinical endometritis compared to estrous cycle (Loyi et al. 2012). Although, the expression was more in the luteal phase of uterine infection, the subclinical and clinical endometritis at midluteal phase was responsible for the significant upregulation of TLR4 and TLR5 transcripts in the bubaline endometrium, using acyclic group as a calibrator (Ajevar et al. 2014). Similarly, the inflammatory status of endometrium (cytological and purulent endometritis) was found to be more often related with upregulation of membrane-bound TLRs (TLR 2, 6 and 10) (Kharayat et al. 2019). During postpartum (pp) period, transcripts of TLR2, TLR4, and proinflammatory cytokines were significantly upregulated on PP day 15 as compared to PP day 60 in the bovine endometrium (Chapwanya et al. 2012).

Variable levels of expression of TLR 1/6, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR10 transcripts were observed in the intercotyledonary region (Martins et al. 2011). It was demonstrated that among all the TLRs, TLR9 has prominent role in attributing host resistance to *B. abortus* infection (Oliveira et al. 2008). In mares, the transcripts of TLR2 and TLR4 were significantly upregulated by 3 h post inoculation of *E. coli* at estrus and diestrus (Marth et al. 2015). Similarly, the transcripts of TLR2 and TLR4 were significantly upregulated during subclinical endometritis compared to chronic endometritis and nonendometritis cases in mares (Siemieniuch et al. 2016). Bitches with cystic endometrial hyperplasia showed upregulation of TLR2 in the uterus (Silva et al. 2010). Similarly, endometrial TLR4 transcripts were upregulated in the bitches with hyperplastic pyometra as compared to atrophic pyometra (Singh et al. 2018). In the ewe, endometrial TLR 2, 7, 8, and 9 were upregulated during the midluteal phase and downregulated during the late luteal phase of the estrous cycle. In addition, their expression remained unchanged between day 10 and 16 of the pregnancy (Ruiz-González et al. 2015). Comparative expression analysis of TLRs in the different organs of the goat by conventional RT-PCR revealed that the uterus had less expression of TLR3, 4, and 10 (Tirumurugaan et al. 2010).

Functional studies on the interaction between endometrial TLR and bacterial PAMPs are largely limited to TLR4, which recognizes the LPS component of *E. coli*. Administration of intravenous *E. coli* LPS results in interference with the estrous cycle by delaying the ovulation, luteogenesis, and rise in P_4 as a result of disrupting the neuroendocrine activity. The LPS-mediated upregulation of caspase-3 during diestrus transiently suppressed both the structure and function of the CL (Herzog et al. 2012). In addition, an association between CL diameter of the first PP cycle and metritis in cows suggests an inimical effect of metritis on PP ovarian function (Williams et al. 2007). Intravenous administration of LPS cannot serve as a model for localized clinical or subclinical endometritis, because it mimics the septic shock induced by toxic puerperal metritis that occur within day 10 PP.

It was found that both endometrial stromal and epithelial cells under in vitro condition produced increasing amount of PGE_2 and $PGF_{2\alpha}$, respectively, in a dose-dependent manner and this response was significantly abrogated by neutralizing LPS with polymyxin (Herath et al. 2006). The peripheral concentration of LPS and PGE_2

was more in cows that suffered with the *E. coli* infection postpartum. Under in vitro conditions, the LPS stimulation of endometrial explants and cells resulted in the preferential increase in PGE_2 rather than $PGF_{2\alpha}$ concentration (Herath et al. 2009). It is well known that endometrial epithelial cells are the exquisite source of $PGF_{2\alpha}$ as they harbor oxytocin receptor and stromal cells produce more PGE_2 (Forteir et al. 2008). However, LPS stimulation of endometrial epithelial cells has shown more accumulation of PGE_2 than $PGF_{2\alpha}$. The increased level of phospholipase A2G6 (PLA2G6) protein in the endometrial epithelial cells was reported to be associated with this switch in PG accumulation, rather than changes in the levels of PGES (PGE synthase) or PGFS (Herath et al. 2009). It would be interesting to know the mechanism of LPS- or infection-mediated switch from $PGF_{2\alpha}$ to PGE_2 in the epithelial cells. The role of endometrial TLRs 2, 1, and 6 in the innate immunity and inflammatory response was investigated in the endometrial cell culture (Turner et al. 2012). It was reported that the triacylated or diacylated bacterial lipopeptides stimulated the production of cytokines IL-6 and IL-8 in the primary bovine endometrial epithelial and stromal cells.

It has been reported that LPS in the follicular fluid decreases the aromatase expression in granulosa cells, increases the secretion of PGE_2 and $PGF_{2\alpha}$, and transcripts of pro-inflammatory cytokine, which results in altered ovarian function (Sheldon and Bromfield 2011; Borges et al. 2012; Jacaa et al. 2013). However, uterine infection does not alter the peripheral plasma FSH concentrations or the consequent follicular wave emergence (Sheldon et al. 2002; Williams et al. 2007). The importance of localized uterine infection on the ovarian dysfunction is interesting as it gives intravenous LPS administration model, a "hormone-like role"; hence, the relative importance of utero-ovarian pathway and general circulation has to be unraveled in relation to uterine infections. In addition to LPS, the other virulence factors of *E. coli* include fimbriae, cell wall proteins, and adhesions, which are putative ligands for various TLRs. The presence of fimH gene in the uterus on PP day 3 has been reported to be associated with increased risk of metritis (Bicalho et al. 2010). Recombinant flagellin, a TLR5 agonist, was not as effective as LPS in stimulating PGE_2 and upregulating proinflammatory cytokines from the bubaline endometrial stromal cells in vitro (Dar et al. 2019). Little information is available on the interaction between PAMPs of *A. pyogenes, F. necrophorum*, or *Provetella sp.* and corresponding bovine endometrial TLRs. The adverse effect of LPS on follicular and CL functions due to its presence in the follicular fluid ushers us to investigate the presence and role of other bacterial VFs in the ovarian compartment. It is well known that myometrial contractions play an important role in eliminating the exudate, so the effects of TLR-PAMP interaction on contraction-associated proteins in the myometrium deserve attention. It is worth mentioning that intrauterine administration of LPS was attempted as a treatment for endometritis in the cattle (Dhaliwal et al. 2001) and buffalo (Singh et al. 2000).

7.6   Hormonal Regulation of TLR Expression

Among all the mucosal tissues, the uterus is exceptional on two accounts: First, ovarian steroid hormones have considerable effects on both afferent and efferent immune events (Wira and Rossoll 1995). Thus, the stage of the estrous cycle (follicular and luteal) at which priming or infection takes place has a marked influence on the outcome of an immune response and stimulation of protective immunity to pathogens. Second, unlike other organs, uterus lacks organized secondary lymphoid aggregates such as Peyer's patch of the ileum (Robertson 2000). In the cattle, luteal phase reduces the endometrial innate immune response and secretory activity of the endometrial glands provides favorable environment for the establishment of pathogens. In contrast to luteal phase, the rising levels of estrogens in the follicular phase increase uterine defense by promoting migration of immune cells into the uterine lumen (Liu et al. 2012). The significant upregulation in the transcripts of TLR2/TLR6, TLR3, 4, 5, 6, 7, and 9 was reported in the endometrium during the secretory phase than any other phase of the menstrual cycle, suggesting that progesterone has a positive influence in the expression of TLRs (Nasu and Narahara 2010). In contrast, inflammatory status of the endometrium rather than the stage of estrous cycle was associated with endometritis in the buffalo (Kharayat et al. 2019). The TLRs are also believed to have physiological role in the female reproductive events (Kannaki et al. 2011) although, they are studied predominantly in relation to uterine infection and endometritis. For instance, it was demonstrated in ewe that the development of ovine endogenous retrovirus, secretion of interferon-tau, and formation of binucleate cells are inhibited by antisense oligonucleotides of TLR7 and TLR8 (Ruiz-González et al. 2015).

7.7   Conclusion

It is concluded that TLRs are expressed in the endometrium of the cow, buffalo, ewe, goat, mare, and bitch. Treatment of endometrial cells with bacterial ligands or synthetic molecules upregulated the expression of proinflammatory cytokines, indicating that the TLRs are functional. Inflammatory status of the endometrium, but not the stage of estrous cycle, appears to regulate the TLR expression. Of all the TLRs, TLR 4 stimulates PGE_2 production when activated by *E coli* LPS, suggesting that endometritis would affect the luteal function by altering the ratio of $PGF_{2\alpha}$ to PGE_2 in the cow. Apart from LPS of *E. coli*, the role of other PAMPs of *E. coli*, *A. pyogenes, F. necrophorum*, and *Provetella sp.* needs to be studied in the context of bovine endometritis as it will expand the role of innate immune response genes in clearing the infection.

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New Insights into the Mechanism of Maternal Recognition of Pregnancy in Ruminants

8

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Abstract

Maternal recognition of pregnancy (MRP) is an early embryonic event that converts a cyclical corpus luteum (CL) into a pregnancy CL. In the ruminants, MRP is mediated by the conceptus-derived trophoblastic interferon-τ (IFNτ) that inhibits the release of oxytocin (OXT) driven luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the epithelial cells of the endometrium. In parallel, IFNτ stimulates the production of PGE2 from the stromal cells that has luteoprotective effects. This chapter attempts to update the mechanisms by which PGE_2 favors MRP and early establishment of pregnancy. Understanding the regulation of the endometrial OXT receptor (OXTR) expression during the late luteal phase of a fertile estrous cycle is an important, but least, understood aspect of MRP.

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8.1 Introduction

With continuous growth in the livestock production, the falling fertility is emerging as cosmopolitan challenge to stakeholders. Reproductive efficiency largely depends on submission rate and pregnancy rate (Sanchez et al. 2018). Conception failure due to early embryonic mortality affects the pregnancy rate and extends the calving to conception interval (Diskin and Morris 2008). Embryo endometrial interaction under the progesterone (P_4) milieu is essential for successful pregnancy (Dey et al. 2004), and paracrine signals from the elongating blastocyst act on the uterus in the establishment of pregnancy in the ruminants. Progesterone and other placental hormones regulate the establishment and sustenance of pregnancy by promoting endometrial differentiation, creating conducive uterine micro-milieu, and mediating the conceptus-uterine interactions (Carson et al. 2000; Paria et al. 2000; Gray et al. 2001).

Progesterone and placental hormones act directly on the uterine endometrium during pregnancy to govern cell differentiation and function, morphogenesis, hyperplasia, and hypertrophy of endometrial glands, rendering increased output of secretory proteins, which are transported to the fetus. Developing conceptus is nourished by histotroph or uterine milk secreted from endometrial glands and appears to be essential for conceptus survival and growth throughout pregnancy in domestic animals (Spencer and Bazer 2004). Progesterone also induces myometrial quiescence and inhibits lymphocytes locally in the ewe (Hafez et al. 2000).

Establishment of pregnancy (ESP) comprises of maternal recognition of pregnancy (MRP) and implantation. Maternal recognition of pregnancy is a phrase that was coined by Short (1969) and is defined as the physiological process whereby the conceptus signals for its presence to the maternal system and prolongs the lifespan of the corpus luteum (CL). Understanding the mechanism of MRP will pave the way forward to develop a therapy intended to rescue the pregnancy losses by improving embryo survival (Spencer and Bazer 2004). Following an infertile estrous cycle, circulating P_4 drops during the late luteal phase due to the pulsatile release of $PGF_{2\alpha}$ pulses from the endometrium of farm animals. In contrast, following fertile mating or insemination, luteolysis is inhibited by the phenomenon of MRP to extend the lifespan of the CL (Raheem 2017). Knowing the process of luteolysis is a prerequisite to understand rescuing mechanism of CL through MRP.

8.2 Mechanism of Luteolysis in Ruminants

Regression of CL is mediated by P_4, estradiol, and oxytocin (McCracken et al. 1999). These hormones induce pulsatile release of $PGF_{2\alpha}$ from the endometrial luminal epithelium (LE) and superficial ductal glandular epithelium (sGE) by acting through their cognate receptors. In addition, Prostaglandin (PG) endoperoxide synthase-2 (PTGS2; also called as cyclooxygenase-2) are also expressed in these cells, which play crucial role in conversion of arachidonic acid (liberated by cytosolic Phospholipase A_2 (cPLA$_2$) from the cell membrane) into PGH_2, the common

intermediate metabolite for all kinds of PGs (Arosh et al. 2015). In early diestrus, P_4 from the recently developed CL promotes accumulation of phospholipids in LE and sGE wherefrom arachidonic acid can be liberated for synthesis and secretion of $PGF_{2\alpha}$ (Boshier and Holloway 1973). Coupling of oxytocin with its receptors activates the PLA_2, COX-2, and PG synthases through activation of Ca^{2+} and protein kinase C (PKC) pathways and thus initiates the biosynthesis of PGs (Lee et al. 2013). The sources of oxytocin are posterior pituitary and CL (large luteal cells). $PGF_{2\alpha}$ release is displayed in either continuous baseline or/and pulsatile pattern, which may depend upon the availability of luteal boost of oxytocin. During estrus and metestrus, PGF pulses are absent due to lack of endometrial oxytocin receptors as well as lack of luteal oxytocin.

High concentration of circulating estradiol during the estrus phase upregulates the estrogen receptor alpha-1 ($ER\alpha$), oxytocin receptors (OXTRs), and P_4 receptors (PRs) in the endometrial cells, whereas P_4, upon coupling with PR, downregulates the synthesis of ER_α and OXTR. During estrus and early metestrus, OXTRs are present on the uterine LE and sGE. Though PRs are also present at this time, but due to basal level of systemic P_4, the activated PRs remain insufficient to suppress $ER\alpha$ and OXTRs syntheses. This hypothesis is strengthened by the observation that administration of P_4 exogenously during the metestrus shortened the cycle length in sheep and cattle by advancing luteolytic process (Woody et al. 1967; Garrett et al. 1988).

As CL grows on, P_4 concentration begins to rise gradually and more PRs get activated and block the expression of $ER\alpha$ and OXTRs in the endometrial LE and sGE during the first few days of diestrus. Hence, the number of $ER\alpha$ and OXTRs is reduced to the undetectable level during Day 5 to 11 of the estrous cycle in the ewe. Later on, constant exposure of the endometrium to P_4 for 8–10 days downregulates the expression of PR gene in the endometrial LE cells by Day 11–13 of the ovine cycle. Depletion of PR results in withdrawal of the P_4 block to $ER\alpha$ and OXTR expression allowing an instant rise in formation of $ER\alpha$ after Day 13, followed by an increase in the expression of OXTR after Day 14 of the cycle (McCracken et al. 1999; Spencer et al. 2004, 2007). This increased expression of $ER\alpha$ and OXTR is facilitated by estrogen secreted by midcycle growing and dominants follicles (Wathes and Hamon 1993; Spencer et al. 1995). Thus, P_4 acts paradoxically first in the suppression and then induction of endometrial luteolytic mechanism during the estrous cycle (Spencer and Bazer 2004). This was inferred from the studies done by treating the cyclic sheep with RU486 (Mifepristone, a PR antagonist) during early diestrus phase, which prolonged the cycle length (Morgan et al. 1993; Johnson et al. 2000), because RU486 blocks the maximum PR and thereby curtails the duration of P_4 exposure on PR. This delays the P_4 mediated downregulation of PR gene expression, and thereby, restoration of $ER\alpha$ and OXTR is also delayed for production of $PGF_{2\alpha}$ pulses (Fig. 8.1).

Both kinds of CL (periodicum and graviditatis) begin to release oxytocin from Day 9 post estrus in sheep; however, sufficient number of OXTR is not present by that time to elicit luteolytic pulses of $PGF_{2\alpha}$. As OXTRs are upregulated by Day 14 of the cycle, available oxytocin pulses from posterior pituitary act on oxytocin
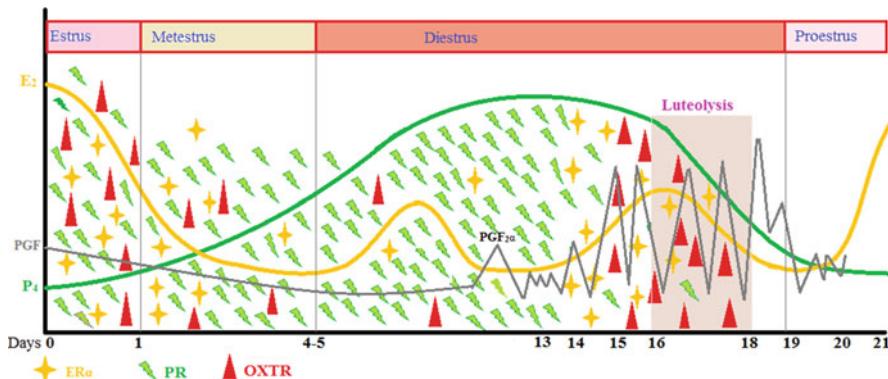
Fig. 8.1 Upregulation and downregulation of ERα, OXTR, and PR under the influence of varying concentrations of reproductive hormones and onset of luteolytic pulses of $PGF_{2\alpha}$ in the cow

receptors in endometrial LE cells, which in turn produce the luteolytic pulses of $PGF_{2\alpha}$ between Day 14 and 16 of the cycle. These luteolytic pulses of $PGF_{2\alpha}$ are amplified by luteal oxytocin, which acts as a supplemental source of oxytocin to boost the oxytocin pulses from posterior pituitary during luteolysis (Spencer and Bazer 2004; Spencer et al. 2007; Arosh et al. 2015; Hansen et al. 2017). Regression of the CL requires at least five $PGF_{2\alpha}$ pulses within 48 h at an interval of 8 h and each wave lasts for 1 h to effect complete luteolysis in sheep (McCracken et al. 2012).

8.3 Transport and Action of $PGF_{2\alpha}$

Endometrial $PGF_{2\alpha}$ enters into venous drainage from the uterus through uterine vein, which runs in close proximity to the ovarian artery that becomes highly tortuous and forms a complex network adhered over uterine vein so-called utero-ovarian plexus (UOP) (Silvia and Niswender 1986). As the direction of blood flow is opposite or antiparallel in uterine vein and ovarian artery, the $PGF_{2\alpha}$ is transported from vein to the artery at a faster rate of diffusion and reach to the ovary (Senger 2003; Arosh et al. 2015). It has been demonstrated that transport of $PGF_{2\alpha}$, which is released in pulsatile pattern, is mediated through PGT (PG transporter), whereas the basal release of $PGF_{2\alpha}$ is conveyed by simple diffusion in the ewe (Banu et al. 2008; Lee et al. 2010, 2013) (Fig. 8.2).

Luteolytic pulses of $PGF_{2\alpha}$ stimulate the expression of COX-2 mRNA in the CL of sheep (Tsai and Wiltbank 1997, 1998; Anderson et al. 2001), suggesting that luteal oxytocin and PGF seem to provide a positive feedback loop in achieving the luteolysis. Production of intraluteal $PGF_{2\alpha}$ is facilitated by activation of luteal PG biosynthetic machinery through Ca^{2+} and PKC pathways in large luteal cells, which are induced upon coupling of PGF receptors with endometrial pulses of $PGF_{2\alpha}$. Luteal $PGF_{2\alpha}$ production is further autoamplified by autocrine and paracrine mechanisms and eventually results in suppression of the survival pathways and
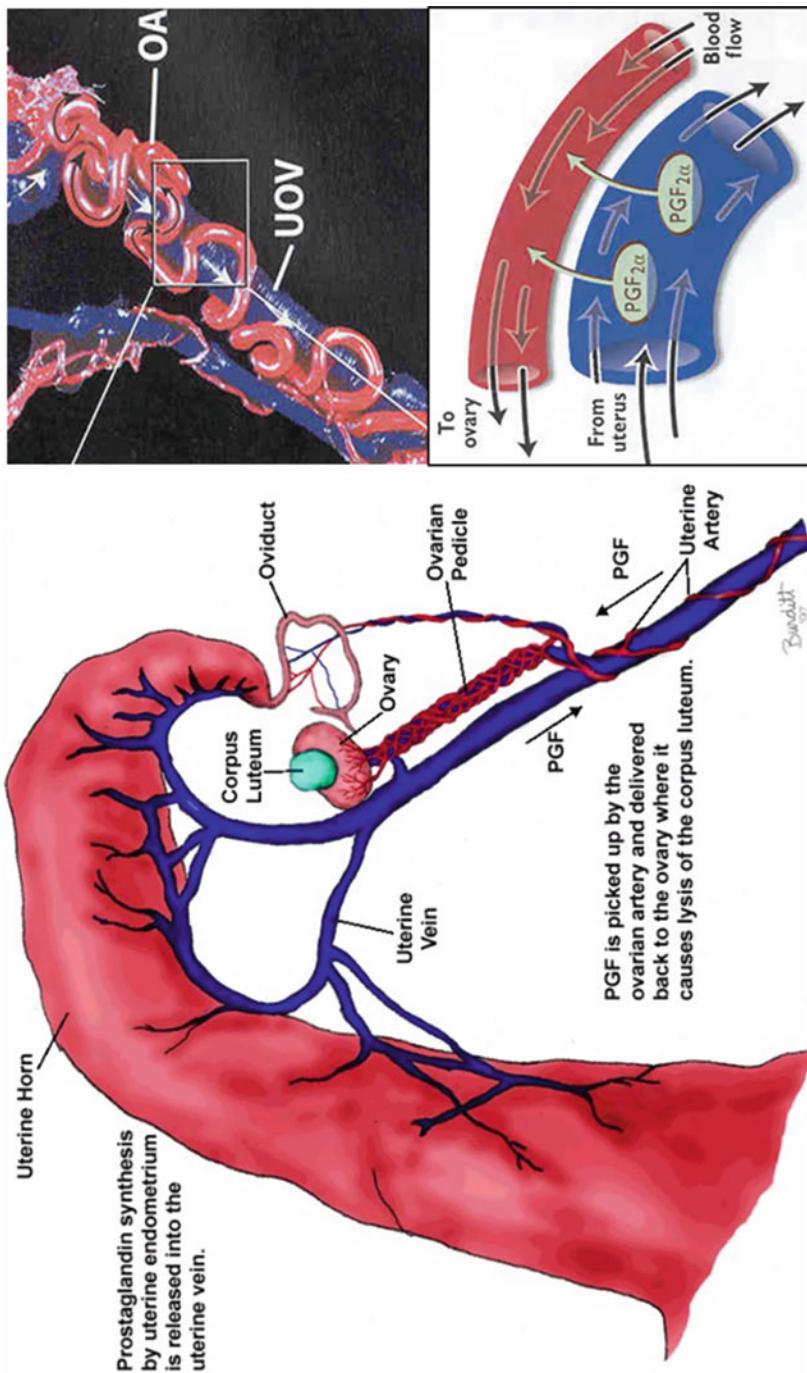
Fig. 8.2 Local transport of $PGF_{2\alpha}$ from uterus to ovary through UOP (Reproduced from Senger 2003)

activation of apoptotic pathways to cause complete luteolysis through various mechanisms viz. vasoconstriction followed by tissue necrosis, local infiltration of macrophages, which may secrete cytokines, such as tumor necrosis factor (TNF)-α, and finally, nitric-oxide-mediated pathway (Noakes 2009).

In vivo and in vitro studies using indomethacin (COX-1/2 inhibitor) to block the PG synthesis in the uterus and/or CL (Guthrie 1979; Rexroad and Guthrie 1979; Sawyer et al. 1990; Zheng et al. 1994; Niswender et al. 2007) revealed that uterine $PGF_{2\alpha}$ causes functional luteolysis, while luteal $PGF_{2\alpha}$ causes structural luteolysis. Functional regression represents the abrupt fall in the circulating P_4, whereas structural regression denotes physical demise and involution of the CL.

8.4 Maternal Recognition of Pregnancy in Ruminants

It is evident that the cyclical CL needs to be rescued by preventing luteolysis for maintenance of pregnancy. $PGF_{2\alpha}$ is luteolytic agent, whereas PGE_2 is the luteoprotective agent as it antagonizes the pharmacological actions of the $PGF_{2\alpha}$. Hence, there are following possible ways by which CL can be rescued:

1. Inhibiting the synthesis of $PGF_{2\alpha}$
2. Hampering the transport of $PGF_{2\alpha}$ from uterus to the ovary
3. Increasing the production of luteoprotective agent PGE_2 by diversion of PG synthesis pathway toward PGE_2 production in endometrium
4. Preferential transport of PGE_2 over $PGF_{2\alpha}$ from uterus to the ovary
5. Preferential synthesis of PGE_2 over $PGF_{2\alpha}$ in luteal tissue
6. Selective catabolism of $PGF_{2\alpha}$ in luteal tissue

Interferon tau (IFNτ), elaborated by the trophoectodermal cells of the elongating conceptus by gestation day 10, is the agent of MRP in the ewe. IFNτ acts differentially on the LE, GE, and stroma cells of the uterus to revoke the luteolytic process as well as to modulate the expression of certain IFN-stimulated genes (ISGs), which are supposed to play key roles in endometrial differentiation and implantation. Thus, the CL of pregnancy is rescued by conceptus itself to sustain the pregnancy by ensuring uninterrupted supply of P_4.

8.4.1 Inhibition of $PGF_{2\alpha}$ Synthesis

Earlier studies proposed that probably IFNτ suppresses the $PGF_{2\alpha}$ production through PTGS2 inhibition; however, endometrial COX2 expression is upregulated during the window of MRP (Charpigny et al. 1997; Kim et al. 2003). In bovine endometrial epithelial cell line, IFNτ upregulated the COX-2 protein and did not downregulate the OXTR expression (Krishnaswamy et al. 2009). Endometrial LE expresses cell surface receptors for type I IFN, which are made up of two subunits, IFNAR1 & IFNAR2 (also denoted as INFR-1 & 2). IFNτ upon binding with the

receptors exerts its antiluteolytic effect by inhibiting the ERα gene transcription in ovine and OXTR gene in both ovine and bovine endometrial LE and sGE (Hansen et al. 2017). Downregulation of ERα and OXTR leads to no production of $PGF_{2\alpha}$ pulses (Stewart et al. 2001; Spencer and Bazer 2004; Wang and Roberts 2004). Moreover, IFNτ does not inhibit the basal production of $PGF_{2\alpha}$ from the endometrium, which is higher in pregnant than the cyclic ewes (Reviewed by Arosh et al. 2015).

8.4.2 Hampering the Transport of $PGF_{2\alpha}$ from Uterus to the Ovary

Though endometrial synthesis of $PGF_{2\alpha}$ is highest on Day 14 to 15 post estrus in both pregnant and cyclic ewes (Hooper et al. 1986; Zarco et al. 1988), IFNτ hampers PGT-mediated transport of $PGF_{2\alpha}$ pulses from the endometrium to CL through UOP. The PGT function is inhibited via ERK1/2 (extracellular signal regulated protein kinases 1 & 2) pathway wherein PGT protein is phosphorylated at tyrosine and threonine residues, whereas serine residue is dephosphorylated in tandem (Lee et al. 2014). Further, IFNτ did not inhibit basal production of $PGF_{2\alpha}$, because it is transported from the endometrium to UOP by simple diffusion without involving PGT-dependent mechanism (Banu et al. 2008; Lee et al. 2013) and is the likely reason behind increased basal concentration of $PGF_{2\alpha}$ during pregnancy establishment (Arosh et al. 2015).

8.4.3 Increasing the Production of Luteoprotective Agent PGE_2 by Diversion of PG Synthesis Pathway Toward PGE_2 Production in Endometrium

IFNτ increased ISG15 mRNA in the ovine endometrium during Day 10–14 of the cycle and the effect was eliminated by coadministration of COX-2 inhibitor, meloxicam (Dorniak et al. 2011). Similar observation was also made in the cow and ewe by Spencer et al. (2013). Antoniazzi et al. (2013) reported that administration of IFNτ into either uterine vein or jugular vein protected CL from the luteolytic actions of $PGF_{2\alpha}$ irrespective of the dose and route, probably through modulating luteal ISG and stabilizing the cell survival genes. The luteoprotective effects of PGE_2 are further supported by the observation that intraovarian injection of PGE_2 counteracted the luteolytic effects of $PGF_{2\alpha}$ (Henderson et al. 1977). PGE_2 is synthesized and secreted by both conceptus (Lacroix and Kann 1982; Charpigny et al. 1997) and endometrial cells in vitro (Marcus 1981; Lacroix and Kann 1982). Administration of PGE_2 in cyclic ewes (Henderson et al. 1977; Magness et al. 1981; Reynolds et al. 1981) and cows (Reynolds et al. 1983) through intrauterine or intraovarian route prolonged the cycle length and reduced luteal sensitivity to both endogenously released and exogenously administered $PGF_{2\alpha}$. Thus, the polycrine actions of PGE_2 seem to favor the establishment of pregnancy (Arosh et al. 2015).

8.4.4 Preferential Transport of PGE$_2$ Over PGF$_{2\alpha}$ from Uterus to the Ovary

PGE$_2$ is a small molecule of 0.35 kDa in weight possessing lipid-soluble property, which is transported locally from uterine endometrium to the CL (Silvia and Niswender 1986) through UOP via PGT-mediated transport (Banu et al. 2003; Lee et al. 2010). A number of previous studies indicated that PGE$_2$ secretion from the endometrium to the uterine vein rises at the time of luteal resistance in early pregnant ewe (Lewis et al. 1978; Silvia et al. 1984; Rawlings and Hyland 1985; Vincent et al. 1986). Lee et al. (2012) tracked the vascular route of PGF$_{2\alpha}$ and PGE$_2$ from the uterus to the ovary and measured the respective quantities in uterine vein, utero ovarian plexus, and ovarian artery during Day 12 to 16 post estrus in cyclic and pregnant ewes. Surprisingly, the uterine concentration of PGF$_{2\alpha}$ was found to be significantly higher than that of PGE$_2$ on Day 16 post estrus in both cyclic and pregnant ewes. However, ~85% PGF$_{2\alpha}$ was transported from the uterus to the uterine vein during the period of luteal regression in the cyclic ewes, whereas in pregnancy, barely ~35% of PGF$_{2\alpha}$ was found to be transported from the uterus to the uterine vein during the window of MRP. Further, PGE$_2$ was transported selectively from the uterus to the ovary at the time of MRP. Concentration of PGE$_2$ was higher in the uterine vein (~30 ng/mL) and ovarian artery (~4 ng/mL) during pregnancy than those of cyclic counterparts where the PGE$_2$ was found to be in similar concentration (~0.5 ng/mL) in the uterine vein and ovarian artery on Day 16 post estrus (Lee et al. 2012). This data indicates that the transport of PGE$_2$ from uterus to uterine vein and then to the ovarian artery depends upon concentration gradient. If it is true, then the PGT-mediated transport of PGE$_2$ is called into question, because concentration-gradient-dependent transport represents the simple diffusion. Therefore, further studies are required to understand the exact mechanism.

8.4.5 Preferential Synthesis of PGE$_2$ Over PGF$_{2\alpha}$ in Luteal Tissue

The endometrial PGE$_2$ upon reaching the ovary interacts with their cognate EP2/EP4 receptors in the large luteal cells and triggers antiapoptotic or survival pathways through cAMP-PKA-SRC cascade and further autoamplifies intraluteal PGE$_2$ production via autocrine and paracrine signaling. As discussed above, the uterine concentration of PGF$_{2\alpha}$ was found to be significantly higher than that of PGE$_2$ on Day 16 post estrus in both cyclic and pregnant ewes. The reverse was observed at luteal level in the same experiment that revealed that the ratio of PGF$_{2\alpha}$ and PGE$_2$ on Day 14–16 of pregnancy was recorded to be lower. Considering the differential transport of PGs from the uterus to the CL and to rule out those uterine fractions at luteal level, the PG biosynthetic enzymes viz. PGF synthase (PGFS) and PGE synthase (PGES) were measured in luteal lysates on Day 14–16 of pregnancy and estrous cycle. The ratio of PGFS to PGES was found to be lower in pregnant ewes, whereas higher in cyclic ewes. Thus, it is inferred that intraluteal synthesis of PGF$_{2\alpha}$ increases on Day 16 of the estrous cycle, whereas intraluteal production of PGE$_2$

rises on day 16 of pregnancy (Lee et al. 2012). Collectively, IFNτ renders the CL resistant to $PGF_{2\alpha}$ through endometrial and luteal PGE_2 via several intracellular mechanisms presumably through preferential biosynthesis of luteal PG diverted toward PGE_2 and protects the CL from luteolysis during pregnancy establishment. And, this event requires overexpression of PTGS-2 protein in the luteal tissue during Day 12 to 16 irrespective of cyclicity or pregnancy (Arosh et al. 2015).

8.4.6 Selective Catabolism of $PGF_{2\alpha}$ in the Luteal Tissue

Bioavailability of PG depends on the balance between synthesis and metabolism; thus, the expression and activity of the catabolic enzyme, PG 15-dehydrogenase (PGHS), needs to be taken into consideration. Lee et al. (2012) reported that PGDH protein is upregulated in the CL of pregnancy during Day 12–16, whereas no alteration in PGDH expression was evidenced during the same period of cyclic CL. Further, concentration of PGFM (inactive metabolite of $PGF_{2\alpha}$) was higher on Day 14 to 16 of pregnancy than that of other days of estrous cycle, whereas PGEM was found to be very low and unchanged. This indicates that intraluteal PGDH protein selectively catabolizes the $PGF_{2\alpha}$ only and does not affect the PGE_2 during pregnancy establishment (Arosh et al. 2015). IFNτ administration upregulates the expression of PGES-1 and intrauterine coinfusion of IFNτ and PGES-1 inhibitor indicated that PGE_2 might mediate the suppressive effects of IFNτ on the expression of ERα and OXTR (Arosh et al. 2015).

8.5 Conclusion

MRP is a complex event initiated by conceptus-derived IFNτ, mediated by functional dyads of $PGF_{2\alpha}$ and $PGF_{2\alpha}$, and regulated by P_4 in the ruminants. Apart from inhibiting the production and release of $PGF_{2\alpha}$ from the endometrial epithelial cells, IFNτ stimulates the production of PGE_2 from the stromal cells and rescues the CL through polycrine actions. Intraluteal synthesis of PGE_2 and increased catabolism of $PGF_{2\alpha}$ also contribute to the sustenance of CL during early pregnancy. Available evidence suggests that both antiluteolytic and luteoprotective mechanisms complement each other in the rescuing the CL from lysis during the window of MRP. Translational research on the role of PGE_2 agonists in minimizing the early embryonic mortality needs to be undertaken.

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Advances in Estrous Synchronization and Timed Breeding Programs for Fertility Enhancement in Cattle and Buffaloes

9

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Abstract

The largest cause of reproductive loss is that the animal fails to become pregnant at a specified interval and/or during the breeding season. Heifers and cows fail to become pregnant, because they do not show estrus (heat) or fail to conceive after showing estrus. The widely accepted techniques like artificial insemination (AI) and estrous cycle synchronization in cattle is a proof of concept that how discoveries in basic science can revolutionize livestock breeding and management. The discovery of ovarian steroids and its potential application through an elaborate research and clinical trial have led to development of methods to control estrous cycle and ovulation in cattle. Controlled breeding results in numerous advantages, which includes elimination of labor-intensive estrus detection at herd level, effective utilization of resources by planned breeding in a short duration of time, reduction in breeding season, production of calf and replacement heifers of same-age group, maximizing the utilization of genetically superior bull through widest coverage of cows through AI. Developing a clear and thorough understanding of the estrous cycle, endocrine control, estrous signs and detection aids, and application of this knowledge in day-to-day clinical practice will not only allow the veterinarian to optimize the reproductive efficiency but also for successful use of estrous synchronization and other reproductive technologies in cattle. This chapter delineates the advances in estrus synchronization and timed AI in bovines.

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9.1 Estrous Cycle

In dairy cattle reproduction, estrous cycle and associated hormones are the cornerstones. In females, once the cow attains puberty, the reproductive cyclicity begins and it continues for lifetime. Estrous cycle is characterized by physiological events occurring internally and associated expression of sexual behavior female occurring between beginning and end of estrus (heat) (Fig. 9.1).

The word "estrus" is derived from a Greek word "Oistros," which means that the behaviors of cows were similar to the behavior when Gad fly attacks cows.

The estrous cycle represents the cyclical pattern of ovarian activity with a successive heat period of 18–24 days. If the cow conceives during heat upon natural/artificial insemination, animal becomes pregnant. During the period of pregnancy, animal enters into physiological anestrus and after parturition, animal enters into normal cyclicity. The estrous cycle is characterized by two distinct phases viz. luteal and follicular phases based on the major hormones and/or ovarian structure. (Fig. 9.2).

Fig. 9.1 Estrous cycle
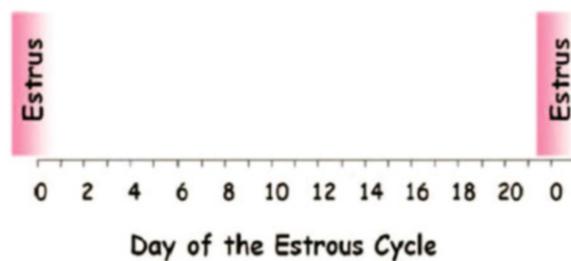
Fig. 9.2 Discrete phases of estrus cycle in Bovine
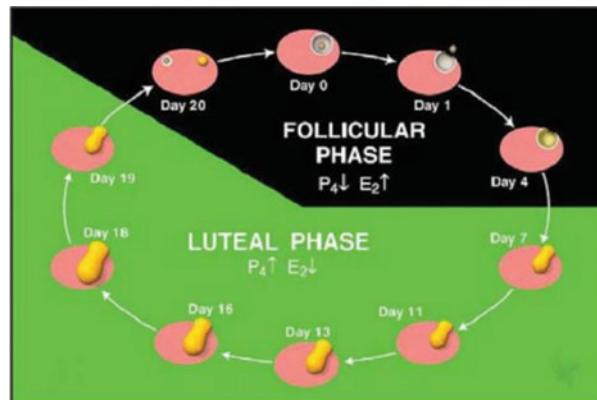
Luteal Phase (14–18 days) This is the longest period of estrous cycle, which occurs between ovulation and subsequent formation and regression of corpus luteum: a temporary endocrine gland, which secretes mainly progesterone hormone. This phase is further divided into metoestrus and dioestrus period.

Follicular Phase (4–6 days) The period following luteolysis (regression of corpus luteum) to ovulation. This phase is characterized by animal expressing heat symptoms culminating into ovulation. This phase is dominated by estrogen hormone, which is secreted mainly from developing follicles/preovulatory follicles.

Subdivisions of the follicular and luteal phases of the cycle are four stages viz. proestrus, oestrus, metoestrus, and dioestrus.

1. Proestrus (2-5 days) is the "building up period" and begins with luteal regression and ends with onset of estrus. This period is characterized by increasing levels of estrogen hormone under the influence of gonadotrophins FSH and LH.
2. Estrus (18–24 h) is the period of sexual receptivity and mating. The main reproductive hormone responsible for estrus behavior and physiological changes in the reproductive tract is oestradiol.
3. Metestrus (3–5 days) is the period from ovulation and formation of CL. It is an ill-defined phase. The cellular transformation of the follicle to the CL is called luteinization.
4. Diestrus (10–12 days) is the longest phase, which is dominated by high levels of progesterone hormone secreted from corpus luteum. During this period, animals do not express heat symptoms and sexual receptivities.

Ultrasound characterization of follicular dynamics reveals that follicle growth occurs in the form of "waves." The bovine follicular dynamics commonly involves 2 or 3 waves of follicular growth during a 21-day cycle (Fig. 9.3a, b). At the beginning of each wave, a cohort of follicles gets recruited under the influence of FSH. From this cohort, few follicles will be selected to grow further and finally only one follicle reaches dominance and becomes a dominant follicle (DF). In the presence of functional CL and progesterone hormone, the dominant follicle lives for a shorter duration (3–6 days) and undergoes atresia (physiological cell death) and the demise of the DF coincides with recruitment of another wave of follicle and the process continues till the development of ovulatory follicle. In the absence of progesterone (due to regression of CL), the dominant follicle of particular wave (second or third wave) reaches to a preovulatory follicle size and culminates in ovulation due to LH surge.

During luteal phase, low levels of LH prevent the follicles/DF from producing high levels of estrogen and ovulation. Around luteolysis, the DF produces high levels of estrogen and elicits positive feedback mechanism to induce ovulatory surge of LH leading to ovulation and in addition high levels of estrogen make the cow to exhibit estrus.

Fig. 9.3 (**a**) Follicular growth occurs throughout the estrus cycle, but estrogen levels only rise during the follicular phase. (**b**) Follicular waves
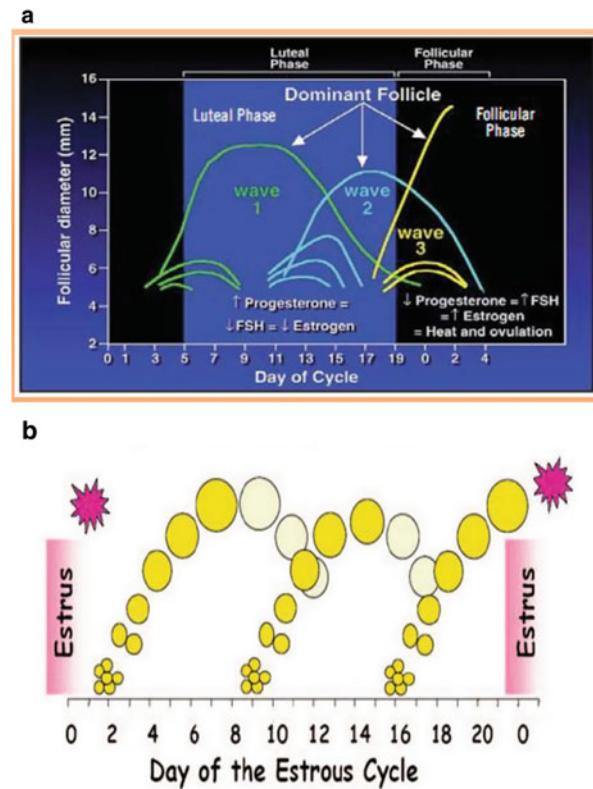
9.1.1 Reproductive Hormones

Dairy cattle reproduction is controlled by several hormones secreted from numerous endocrine glands and are discharged into the blood, thereby reaching their respective site of action for their destined function.

Gonadotropin-Releasing Hormone (GnRH) GnRH acts on the pituitary gland to release follicle-stimulating hormone and luteinizing hormone, which is controlling the various process of follicular development viz. recruitment, growth, and ovulation of the dominant follicle, respectively.

Follicle-Stimulating Hormone (FSH) Growth and development and function of the follicle is regulated by FSH, which is secreted by the pituitary gland (Fig. 9.4a).

Estrogen secreted by the Graafian follicle is responsible for onset of behavioral estrus, induction of ovulation, and the increase of vaginal mucous secretion (Fig. 9.4b).
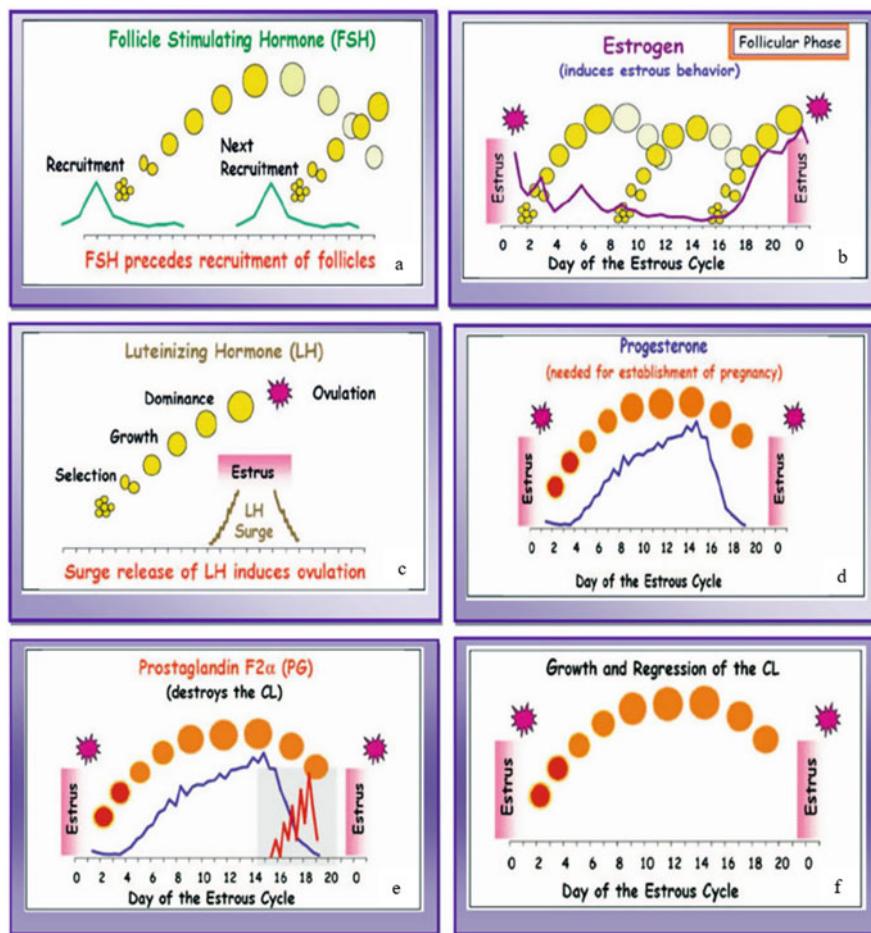
Fig. 9.4 Role of major hormones viz. (**a**) FSH; (**b**) Estrogen; (**c**) LH; (**d**) Progesterone; (**e**) Prostaglandin in follicular wave development and regulation of estrous cycle. Figure 9.4f shows the growth and regression pattern of corpus luteum throughout estrus cycle in bovine

Luteinizing Hormone (LH) This hormone is responsible for ovulation of dominant follicle and responsible for development of CL and also elicits luteotrophic effect (Fig. 9.4c).

Progesterone is secreted by the CL and is essential for pregnancy. This hormone suppresses the LH secretion, thereby preventing the cow from coming into heat and ovulating when pregnant (Fig. 9.4d).

Prostaglandin (PGF2 alpha) secreted by the uterus is responsible for luteolysis. In pregnant cows, pregnant, the embryo will block the release of PGF2 alpha and the

CL to continue to secrete progesterone (Fig. 9.4e). The growth and regression pattern of corpus luteum throughout estrus cycle in bovine is shown in Fig. 9.4f.

9.1.2   Endocrine Regulation

In cattle, estrous cycle occurs every 21 days and the hormones of hypothalamo-pituitary-ovarian axis controls estrous cycle. The various hormones mainly regulate the ovarian and reproductive functions through positive and negative feedback mechanism. GnRH is released from hypothalamus and reaches pituitary gland via the hypophyseal portal blood system and causes release of FSH and LH. The secretory granules in the cytoplasm store FSH and LH for short and long periods of time, respectively.

The progesterone level during the follicular phase of the estrous cycle reaches basal concentration due to luteolysis. Alternatively, estrogen concentration, from the preovulatory dominant follicle (DF), increases and allows the cow to express the estrus behavior. LH surge, concomitant with the basal progesterone, causes the ovulation of DF, which occurs 10–14 h after the end of estrus. This results in the formation of CL and beginning of the luteal phase, which is dominated by progesterone hormone. During the dioestrus phase, P4 concentrations remain elevated and FSH from the anterior pituitary initiates recurrent waves of follicle development. However, these DFs do not ovulate due to the suppressive effect of progesterone on LH surge release (i.e., negative feedback on LH/less frequency pulses of LH). (Figs. 9.5 and 9.6).

Toward the end of luteal phase, that is, from day 16–18, the major luteolytic hormone PGF2α secreted by the uterus is transported to ovary through countercurrent exchange mechanism where it elucidates luteolytic mechanism. Luteolysis results in reduction in circulating progesterone concentrations. The dominant follicle present around this time reaches maximum size of preovulatory stage with concomitant increase in estradiol concentration culminating in estrus and ovulation.

Fig. 9.5 Ovarian changes and endocrine pattern during oestrous cycle in cows
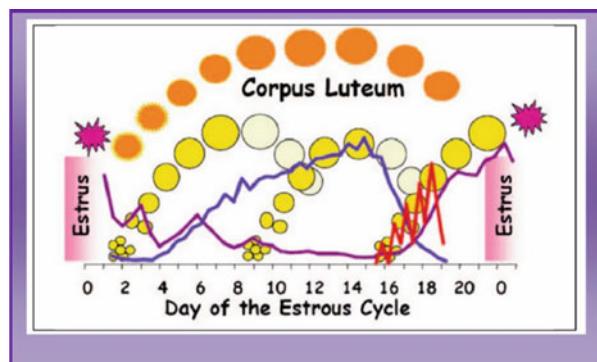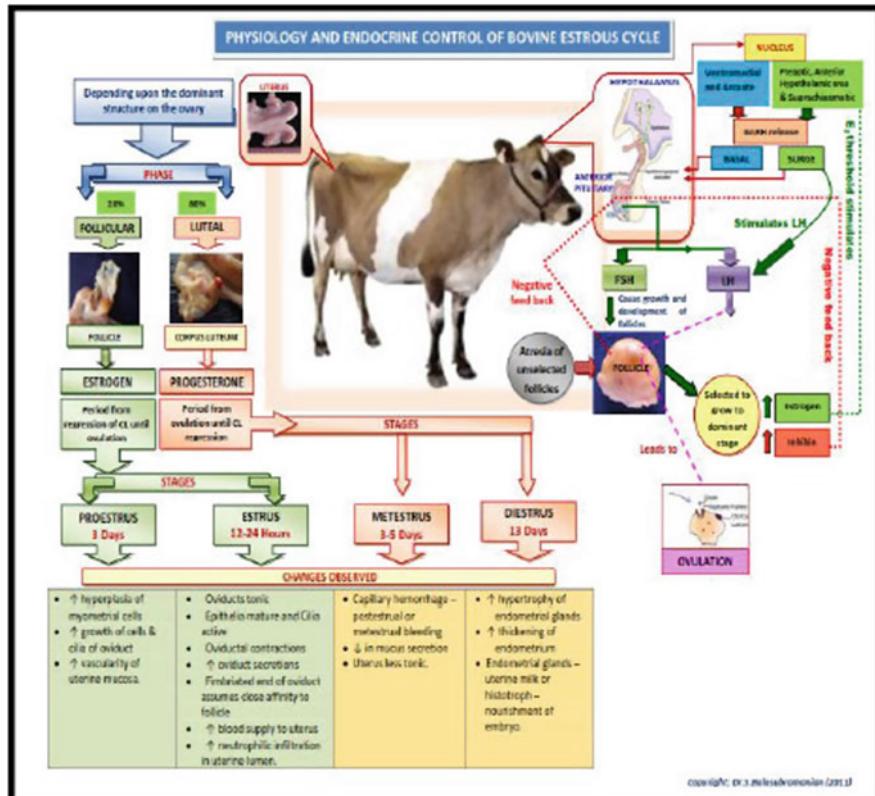
Fig. 9.6 Endocrine control, phases and stages of oestrous cycle including its behavioral events in cattle

9.2 Estrus Signs and Detection Aids

Sound reproductive management is the foundation of successful dairy industry. Breeding close to the time of ovulation is an important strategy, which is possible by effective estrus detection.

9.2.1 Estrus Signs

During estrous cycle, each cow exhibits signs of estrus for a certain period of time. Slight variations exist in the duration of estrus and time of ovulation between cow and buffalo (Table 9.1).

Table 9.1 Reproductive characteristics and duration of events in cow and buffalo

Species	Nature of estrous cycle	Length of estrous cycle (days)	Duration of estrus (h)	Ovulation	
				Type	Time
Cow	Polyestrus	19–23 Average: 21	6–30 Average: 18	Spontaneous	12 h after end of estrus
Buffalo	Polyestrus	17–26 Average: 21	5–27 Average: 16	Spontaneous	14 h after end of estrus

Fig. 9.7 Oestrus signs in cattle

Signs of Estrus Primary signs of heat involve, a cow stands to be mounted during the most sexually intensive period of the estrous cycle and is the most accurate sign of estrus. Cows that are moving away quickly on mounting are not in true estrus. Standing to be mounted for a longer time that is around 3–7 and with more frequency (20–55 times) indicates the right time of heat period. The mean duration of estrus is 15–18 h, but may vary from 8 to 30 h among cows. The behavioral pattern during the heat period is associated with elevated levels of estrogen hormone, which in turn acts on the brain to elicit the behavioral changes associated with standing heat. Secondary signs of heat vary in duration and intensity and may occur before, during, and after standing heat and generally are not associated with ovulation. Cows exhibiting secondary signs must be watched more closely for standing behavior (Fig. 9.7).

Mounting on Other Cows Cows approaching heat or in heat exhibit mounting activity and need close monitoring for standing heat.

Mucus Discharge Clear highly viscous long elastic strands of (glass rod like) mucus discharge from vulva is often seen. The mucus discharge can also be observed coming out while palpating reproductive organs during per-rectal examination. Cows should be observed for presence of smears of mucus on perineal area, thigh, flank, and tail region.

Swelling and Reddening of the Vulva The estrus cow vulva appears moist and swollen compared to pale and dry vulva during midcycle.

Bellowing, Trailing, Chin Resting Cows in estrus are more restless and alert to their surroundings. And cows in "proestrus" and "estrus" persistently trail behind and try to mount other cows. Cattle may bellow frequently during estrus. The cows often rub their chin on the back and the pressure applied during this activity is considered as a sign of receptivity in estrus cows.

Cow approaching heat shows other signs like sniffing, licking of genitalia followed by raising of head and curling of upper lips (Flehmen response) and observed generally after urination by a cow in heat. Milking cows in heat show drop in milk yield, restlessness, and reduced feed intake.

Metestrous Bleeding The bloody mucus discharge observed in the cows recently detected for estrus within 1–3 days is called metoestrus bleeding. This is due to estrogen withdrawl and does not relate with pregnancy success.

Prior to ovulation, behavioral changes in cow occurs due to hormonal variations and "Standing to be mounted" by another cow are considered to be the most prominent and appropriate sign of oestrus.

9.2.1.1 Signs of Estrus in Buffalo

The buffaloes are less overt in exhibiting behavioral estrus compared to cattle. However, estrus behavior is more pronounced during night and early morning hours. Further there is a seasonal influence on buffalo reproduction due to which they enter a period of sexual inactivity from March to June with only 3% of the heats being detected. The peak season for estrus detection in buffaloes is from October to February.

- Homosexual behavior among female buffaloes is rare.
- Heterosexual behavior and standing to be mounted by a bull are the most reliable signs of estrus in buffaloes.
- Other physical signs of estrus such as bellowing, restlessness, vulval swelling, clear mucoid vulval discharge, spontaneous milk letdown, and raised tail are less prominent.
  - Restlessness
  - Bellowing and frequent urination

9.2.1.1.1 Estrus Detection Problems

Missed or Unobserved Estrus
- If in most herds essentially all cows are cycling normally, why does the dairy farmer have difficulty "catching the cycling cow"? There are several reasons: some represent "people problems," but there are several "cow factors" that make estrus detection difficult. The major factors contributing to poor heat detection efficiency are as follows:
  - Failure to spend sufficient time on a daily basis for oestrus detection.
  - Most mounting activity occurs at night in loosely housed herds.
  - Heat periods are short.
  - Low levels of estrus activity when few cows are in heat. This can be a significant problem in small herds and in groups of cows in large herds in which many cows are either pregnant, not cycling, or in the luteal phase of their estrous cycles.
  - Mounts last 10 seconds or less. Farmers must concentrate on estrus detection and should avoid combining it with other activities.
  - Feet and leg problems, slippery floors, summer heat, winter cold, and other environmental factors reduce estrus activity.
- Heat detection programs that limit the effects of these "people" and "cow" factors must be developed in order to maximize heat detection efficiency.
- Estrus detection errors.
- Estrus detection errors must be avoided. Breeding pregnant cows can result in abortion. Breeding nonestrus, open-cows leads to waste of time, semen, and money.
- In order to reduce the number of errors, potential causes of the problem must be identified. In most herds, errors result from the following:
  - Misidentification of cows.
  - Misuse or misinterpretation of the signs of heat.
  - Misuse or misinterpretation of heat detection aids.
  - Cows transmitting the wrong signals (up to 10% of pregnant cows may stand to be mounted.)

The heat detection should be performed when cows are in groups and are in a relaxed state for exhibiting their behavior. The cows should not be observed for estrous signs in restrained, stressful times including milking and feeding. It is best to observe for heat during the coolest time of the day like early morning and late evening. Various factors like housing arrangement, milk yield, floor space, hoof and leg problem, and status of herd-mates affect estrus expression.

9.2.1.1.2 Estrus-Related Phenomena
Various estrus-related physiological or pathological events like metestrus bleeding, split estrus, silent estrus, gestational heat, frequent estrus/nymphomania due to cystic follicle and short cycle (7–10 days of duration) are commonly associated with

estrus-related events. However, these all events need to be differentiated from normal and regular estrus signs.

9.2.2 Estrus Detection Aids

9.2.2.1 Visual Observation

Oestrus detection efficiency by visual observation of standing heat alone is 50–90%. The observation of other secondary signs of oestrus thereby receives significance. The cattle must be observed for estrus two to three times in a day at regular intervals, and typically involving early morning and late evening for better results. Each heat detection should last at least 30 min or sufficiently long. Frequent observation for estrus can improve estrus detection efficiency particularly when estrus is exhibited for shorter duration or of less intensity. To detect standing heat, several aids are available for use; however, none are considered substitutes for visual observation. A dairy farmer or herdman to detect a cow in estrus, largely, relies upon his experience, and the time spent and frequency of observation in a day has a significant influence on estrus detection efficiency. Obviously, a planned and organized way of record maintenance in estrus is also a crucial in dairy farm management.

Above and beyond visual observation of estrus, various methods/devices given below can aid in improving estrus detection in cattle (Tables 9.2 and 9.3).

9.2.2.2 Palpation of the Reproductive Organs

Routine rectal examination of all cows between 30 and 40 days after calving and of individual problem cows that have not been inseminated within 70 days after calving should be encouraged to confirm that the reproductive tract is normal and to predict the next estrus or to identify cows for prostaglandin treatment when estrous cycles are occurring, but estrus undetected.

9.2.2.3 Breeding Wheels, Wall Charts, Herd Monitors, and Individual Cow Records

These other inexpensive methods aim at detection of the next heat period. If the farm workers know when the next possible heat period is, they could closely observe individual cows for signs of estrus. Thus, more short- or weak-heat periods can be identified. Proper recording of heat in postpartum cows and their daily monitoring to identify those cows that are due to return to estrus is very important to reduce the calving interval.

9.3   Estrous Synchronization in Cattle

Estrus synchronization refers to the effective manipulation of length of estrous cycle and ovulation time by which large group of females can be induced heat and inseminated in short predetermined time. In large dairy farms, oestrus detection and animal identification have become increasingly difficult, since most of the time

Table 9.2 Use of teaser animals for heat detection

Teaser bull	Method	Merits	Demerits
Penile-blocked bull	Device installation can be done by veterinary surgeon	• Mounting is normal • Prevents extension of penis and breeding. • Transmission of venereal disease is preventable	• The sexual behavior or drive tends to reduce after a period of 1 year • Not frequently available
Vasectomized bull	Using surgical method, the vas deferens is cut so that the sperm from testes is not carried to penis during ejaculation	• Libido is maintained • Utilization of these bull results in establishing breeding stimuli, which may improve conception rate in cows	• Risk of spread of sexually transmitted diseases
Bull with deviated penis	Deviation of prepuce/penis to one direction prevents entry or direction of penis toward vula/exyernal genitalia while mounting	• In comparison to vasectomized/penile blocked bull, this method is better • no chances of transmission of sexually transmitted diseases and loss of sexual desire	• Accidentally some bulls may copulate
Caudal - Epididymectomy	Tail portion of epididymis is removed so that sperms do not reach the penis while ejaculation	• This method is easy to perform and economical	• chances of transmission of sexually transmitted diseases
Androgenized female	In this method, prior to breeding season, testosterone propionate injections is given or Synovex® H ear implants (200 mg testosterone propionate/20 mg estradiol benzoate)	• This method is safe than using bulls • Economical	• Response to treatment can vary. • Usage may not be possible with all country and may depend on every country's ethical guidelines. • Availability of drugs (import restrictions) and cost

Source: J. A. Parish (2010), Mississippi State University (presented with little modifications)

we need to depend on the animal handler's/milker's information and less time for interaction. In intensive dairy farming, the cows show less intense oestrus signs for short duration of time. The likely expression of the classic symptoms of behavioral estrus is limited due to comparatively small body size and less plasma estrogen concentrations from higher metabolism.

Table 9.3 Common estrus detection aids in cattle

Detection aid	Principle	Application	Management considerations
Kamar heat mount detector	When the pressure is exerted on this device by the mounting animal, the color of the device changes from white to red	The device is applied over the sacrum region or above tail head area by using an adhesive	Dislodgement of device, partial activation of device
Estrotect-heat detector	Detector remains silver until friction of mounting animal(s) reveals fluorescent color under scratched-off silver layer	Apply with self-adhesive between tail- head and hipbone over the sacrum of cow or heifer	Low branches, gates, and other cattle can lead to a false positive. Detectors can become dislodged from female
Bovine Beacon®	When a cow or heifer in heat is mounted by another animal, the fluorescent dye will glow in the dark	Glue to tail-head of cow or heifer	Low branches, gates, and other cattle can lead to a false positive Dislodgement of detector
Tail-head markers	When marker is rubbed off tail-head (hair will be ruffled and pulled back), the cow or heifer has stood to be mounted	Smear liberal amounts (at least 2–3″ wide) of crayon, chalk, paste, or paint marker on the tail-head of cow or heifer	Low branches, gates, other cattle, humidity, and rain can lead to a false positive. May need to reapply every few days
Chin-ball marker	When a cow or heifer in heat is mounted by an another cow, wearing this device slides down from back and hip area and leaves an ink mark	This marker device is fixed below the chin area of a teaser bull/androgenized female	Maintenance, i.e., ink must be refilled regularly. Breakage or stretching of harness straps. Sometimes, due to simple resting of chins may leave marking and gives false information
Heat watch® II	Whenever animal mounts, data are sent from that transmitter to a small radio receiver (base station) in the proximity of the heat detection area. Complete data is generated on every mount	Place small, digital radio transmitter in a piece of polyester material (patch) and glue onto the tail-head of cow or heifer	Patches can become dislodged from females. Transmitters can fall out of patches if not adequately secured. Batteries must have proper charge. Increased heat detection accuracy over other aids
Pedometer	Since cows on heat are generally more restless than usual, the device will pick up increased activity compared to the	These devices are attached to the leg of a cow to monitor activity	Expensive

(continued)

Table 9.3 (continued)

Detection aid	Principle	Application	Management considerations
	average for that cow and the herd as a whole on a particular day, indicating possible heats		
Vaginal electrical resistance	Electrical resistance (ER) of vaginal fluids decreases during proestrus and through the estrus due to increase in the volume and ionic composition of the cervical and vaginal fluids	Estrus probe is designed to monitor these changes, wherein "low" probe readings are associated with estrus	Labor intensive, since cattle must be probed frequently to detect significant changes in ER. Wash the probe in disinfectant and thoroughly rinse and dry it before using in another cow

Various estrous cycle control methods have been practiced to facilitate the breeding of large dairy herd. Commonly, these methods include various protocols utilizing the following agents viz. prostaglandins (PGs), progestins, GnRH, and estradiol.

9.3.1 Development of Methods to Synchronize Estrus

Advancement of research in the area of controlled breeding resulted in two classic approaches to control the cycle length:

1. Induction of luteolysis before natural regression of the corpus luteum (CL) leads to shortening the cycle.
2. Administration of exogenous progestins results in extension of estrous cycle length.

Both the approaches control the fate of CL to decrease or increase the length of the estrous cycle.

Development of estrous synchronization methods in cow has occurred in five distinct phases. The discovery that progesterone inhibits preovulatory follicular maturation and ovulation forms the physiological basis for estrous synchronization.

Phase I: Extending the luteal phase by exogenous progestins.
Phase II: Combination of progestational compounds with estrogens or gonadotropins.
Phase III: Use of luteolytic agents like prostaglandin F2 alpha.
Phase IV: Use of progestational drugs with PG.

Phase V: Manipulation of both follicular waves and luteal life span leading to accurate control of estrous cycle and ovulation.

Phase VI: Use of an aromatase inhibitor for ovarian synchronization in cattle.

9.3.2 Advantages of Synchronization

- Heat detection is one of the most difficult processes in a farm where breeding has to be adopted; synchronization bypasses heat detection and breeding individually as AI can be done at fixed times.
- Breeding seasons can be shortened from 60 to 45 days or less in heifers.
- AI and embryo transfer are made easier.
- Synchronizations allows larger use of elite sires through artificial or natural service.
- Allows breeding to be under a strict timetable.
- Allows batched calving, so that there can be an increased supervision and a more uniform calf crop.
- Allows
  convenient application of fertility treatment to cows, for example, progesterone supplementation during early pregnancy.
- Synchronization would reduce the breeding period to less than 5 days, in a 21-day period cycle.
- It can be an effective management tool when applied correctly.

9.3.3 Criteria for Successful Synchronization

9.3.3.1 Animal Requirements

Disease free—specifically of the reproductive tract
Heifer maturity—proper weight >200–250 kg
Adequate nutrition—must be in positive energy balance
Adequate postpartum interval: 45–60 days
For prostaglandins—animals must be cycling
Normal nonpregnant reproductive tract

9.3.3.2 Management Requirements

Establish program objectives.
Proper timing of activities in the program.
Good records.
Good semen quality.
Good breeding techniques.
Good working facility.
Coordination of all people.
Attention to details.

9.3.4   Hormones Used

Prostaglandin (PGF2 alpha; PG) is a commonly used injectable preparation for synchronization in cyclic heifer and cow. PG can be used as a single hormone for synchronization or in can be used in combination with various other synchronization protocols. Care should be taken before administering PG injection; that is, we need to ensure that the heifer/cow is nonpregnant.

Dose: 25 mg *(Natural)*; 500μg *(Synthetic) Route of administration*: Intramuscular.

Progestins are a synthetic form of progesterone, which is responsible for preparation and maintenance of pregnancy. Administration of a progestin inhibits LH frequency and thereby prevents follicular maturation, estrus, and ovulation. It is commonly used in the form of intravaginal devices (CIDR; Controlled Internal Drug Release) and used in combination with estradiol benzoate to effectively synchronize estrus in cattle and advance the first pubertal estrus in heifers. The CIDR is designed in a flexible "Y"-like contour with nylon thread at tip (for easy withdrawal by pulling). The flexible wings on both sides are concealed with progesterone-loaded silicone skin. The wings are flexible such that it can be collapsed and applied intravaginally using an applicator, and the end of the spine is secured with a nylon thread to facilitate removal. The CIDR device is placed in the vagina for particular period of time, based on the synchronization protocol chosen. The blood progesterone concentrations rise rapidly upon insertion and maximum concentration occurs in an hour and thereafter the progesterone concentration in blood is maintained in a sustained manner generally for 7 days or as long as the device is kept intravaginally. Upon removal of CIDR device, progesterone concentrations rapidly fall as they are quickly metabolized and eliminated.

The CIDR has excellent retention rate of about 97%. The presence of CIDR may cause mild inflammation on the site of application and the vaginal mucus may be clear, cloudy, or yellow but does not have an impact on pregnancy rate. Packaging guidelines should be followed strictly while handling the device.

Dose: 1.9 g/1.38 g of P4 per CIDR insert
Route of administration: Intravaginal
Gonadotropin-Releasing Hormone (GnRH): GnRH causes elevated pulsatile surges of FSH and LH. Injectable preparations are given at a particular time depending upon the chosen estrus synchronization protocol. Administration of GnRH causes ovulation of dominant follicle/preovulatory follicle.
Dose: Gonadorelin: 50 or 100μg**;** Buserelin**:** 10μg.
Route of administration: Intramuscular.

Estrogen: Injectable hormone administered to cause ovulation of follicle with preovulatory capacity by stimulating hypothalamus to release GnRH and subsequent release of LH from pituitary.

Dose: 1 mg (0.5 ml) of Estradiol cypionate (ECP); 1 mg of Estradiol benzoate.
Route of administration: Intramuscular.

9.4   Approaches for Controlled Breeding

The basic approach for estrus synchronization or controlled breeding is by controlling the estrus cycle duration, that is, onset of estrus either by shortening or lengthening the cycle using following approaches.

1. *Shortening the luteal phase*: Administration of natural or synthetic prostaglandin or its analogue to lyse the corpus luteum, that is, by removing the luteal (progesterone) phase of the cycle and thereby bringing animal into estrus (heat). This approach is highly suitable in cycling animals.
2. *Lengthening the luteal phase*: Administration of natural progesterone/synthetic progestins to extend the existing luteal phase or mimic the luteal phase to suppress the dominant/preovulatory follicle development for a stipulated period. This approach is suitable in both cyclic and anestrus animals.
3. *Manipulating onset of follicular wave and ovulation*: This is the most recently developed approach wherein GnRH and Prostaglandin F2 alpha is used at a particular interval of the cycle to control the length of cycle, and program the emergence of follicular wave and occurrence of ovulation. This approach is most suitable in cyclic females and also used in anestrus females to initiate emergence of new follicular wave and estrus cycle.
4. *Use of an aromatase inhibitor for ovarian synchronization in cattle*: Estrogen-based protocols precisely control follicle development and ovulation in cattle that enable precise ovulation, and this approach facilitates the effective management of time, labour, and resources by adopting timed AI. Letrozole is a nonsteroidal aromatase inhibitor that binds reversibly to the "heme" group of the P450 subunit of aromatase enzyme, which is responsible for estrogen synthesis. Irrespective of the day of the estrous cycle, letrozole prolongs the lifespan of the dominant follicle by reducing its rate of growth and estrogen production and thereby ovulation, achieving synchrony.

9.5   Protocols for Estrus Synchronization

Estrus synchronization protocols differ in the products used (hormones, enzyme inhibitors), method of administration (oral, injection, implants, or intravaginal insert), number and timing of injections, handling time, cost of treatment, and heat detection requirements.

Estrus synchronization protocols either recommend timed AI without need for estrus detection or AI preferably at 12 h after detection of standing heat. The cows should be examined at least 5–6 h per day when expected to exhibit heat and peak estrus activity is usually exhibited at 48 to 72 h after PGF2α administration. Those nonresponding cows (without obvious heat signs) are subjected to timed AI 72 to 84 h post PGF2α treatment with a shot of GnRH at the time of AI, which is commonly referred as "cleanup timed AI". The cows, which exhibit obvious heat

signs earlier, will be benefitted by heat detection, whereas the timed insemination of nonresponders can avoid labor-intensive and time-consuming heat detection.

9.5.1   Prostaglandin-Based Protocols

Prostaglandin F2 alpha and its synthetic analogs effectively induce luteolysis in the presence of a responsive CL, that is, day 5 to 7 of the estrous cycle in heifers and 7 to 17 of the estrous cycle in cows. However, CL responsiveness to $PGF_{2\alpha}$ is greatest in late (Days 14–19; 95.7%), intermediate in middle (Days 10–13; 86.4%) and least for early (Days 5–9; 76.9%) stages of the estrous cycle. The stage of follicle development during the $PGF_{2\alpha}$ administration determines the duration to estrus induction as cows with mature follicles enter estrus sooner than cows with immature follicles at the time of treatment. To ensure the presence of responsive CL, two-dose $PGF_{2\alpha}$ protocols were developed. The protocol has the advantage that it can be recommended to cows at unknown stage of estrous cycle as the second $PGF_{2\alpha}$ administered after 7, 11, or 14 days of first injection will be acting on a functional CL and hence, improved response over single $PGF_{2\alpha}$ protocols is obtained (Sabo et al. 2008; Stevenson et al. 1989; Pal and Dar 2020).

Higher plasma progesterone concentrations or exogenous supplementation of progesterone preceding the second dose of PG have been shown to increase pregnancy and submission rates. Alternatively, supplementation of norgestomet injection in the absence of CL prior to a second dose of PG decreases pregnancy rates per AI due to the prolonged dominant preovulatory follicle. Pregnancy rate per AI is reduced as the interval between emergence of ovulatory follicle to estrus and from dominance to oestrus increases. Few follicular waves result in prolonged dominance when plasma progesterone concentration is below 3 ng/mL. Threshold concentrations of progesterone therefore exist, above which progesterone supplementation does not improve fertility more likely due to occurrence of prolonged dominant preovulatory follicle associated with poor fertility.

The $PGF_{2\alpha}$-based protocols for estrous synchronization are outlined in Fig. 9.1. Two dose PG protocol administered at 7 days' interval synchronizes estrus without the need for the detection of CL at the time of treatment. Single-dose PG protocols rely on strategic administration of PG to cows on confirmation of presence of CL followed by estrus detection and AI. Though the protocol reduces hormone cost requires estrus detection over a longer period of time for effective breeding on induced estrus.

9.5.1.1 Corpus Luteum (CL) Regression in Cattle

The bovine corpus luteum (CL) is characterized by an initial rapid decline of progesterone (P4) secretion known as *functional luteolysis*. The morphological luteolysis is characterized by gradual involution of the luteal cells in the ovary, resulting in the formation of connective tissue scar known as *corpus albicans*. However, the structural and functional luteolysis are not well defined and demarcated.

Table 9.4 Proposed model for the differential response of the early and midcycle CL to PGF2 alpha administration in the cow

After PG injection, PGF2α enters into the CL via bloodstream	
In the early CL	In the midcycle CL
• PGF2α acutely stimulates vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF2), and insulin-like growth factor (IGF-II) expression in the bovine CL • VEGF and FGF2 stimulate luteal angiotensin II (Ang II) and PGF2α, and Ang II stimulates luteal PGF2α • This local positive feedback system between angiogenic and vasoactive factors supports angiogenesis and P4 secretion within the CL; consequently, the early CL exhibits resistance to the luteolytic effect of PGF2α • Additionally, PGF2α acutely suppresses endothelin 1 (EDN1) and endothelial nitric oxide synthase (eNOS) expression in the bovine early CL, probably contributing to no response in blood flow	• PGF2α suppresses VEGF and FGF2 expression and creates the increased ratio of angiopoietin ANPT2/ANPT1 (an index of instability of vessels), initiating angiolysis within the CL • PGF2α decreases IGF-I and IGF-II expression directly causing a reduction in P4 • A luteolytic dose of PGF2α stimulates nitric oxide (NO) production by eNOS and inducible nitric oxide synthase (iNOS) expression in the CL; thus, the luteal blood flow is acutely increased as a result of vasodilation by NO action • Coincidently, vasoactive factors such as EDN1, Ang II, and luteal PGF2α start to increase within the CL to induce severe vasoconstriction • These events are coordinated and induce directly or indirectly the drastic decrease in P4 secretion

The secretory function of the CL is intricately regulated by several regulatory factors released from CL and other parts of reproductive tract. The PGF2α released from the uterus at the late luteal phase is the principal luteolytic factor involved in determining the length of estrous cycle. However, the exogenous PGF2α is highly effective in inducing luteolysis when administered at mid-luteal phase.

The action of main luteolytic factor PGF2 alpha is further enhanced by the products of accessory luteal cells such as endothelial cells, immune cells, pericytes, and fibroblasts. The reason behind the early CL being resistant to the luteolytic action of PGF2 alpha is the most fascinating question revolving around CL function and is being addressed (Table 9.4).

In ruminants, angiogenic factors such as FGF2 and VEGF have a crucial role in CL development. Additionally, the luteolysin PGF2 alpha has a stage-specific action depending on the stage of luteal development (early vs. mid) during the estrous cycle in the cow. Taken together, PGF2 alpha appears to possess a dual function, acting as a luteotrophic or an antiluteolytic factor by stimulating angiogenic factors in the early CL but acting essentially as a luteolytic factor by stimulating vasoactive and PG-related factors and by inhibiting angiogenic factors, in the midcycle CL.

Targeted Breeding Program improves the reproductive efficiency of dairy herds in which the cows are treated on the same day of the week, to facilitate AI during following weekdays.

9.5.1.2 Onset of Estrus and Fertility Rate on the Use of Prostaglandins

Low synchronization of estrus is the major limiting factor on the use of timed insemination in single prostaglandin protocol. The time of estrus onset depends on the stage of follicular wave development, follicular populations, size, and maturity/ growth phase of the dominant follicle present at the time of PGF2α injection (Kastelic and Ginther 1991). Further, the estrus response was found more pronounced when PGF2α is administered in the presence of large follicles (King et al. 1982; Stevenson et al. 1989; Tanabe and Hann 1984). Fertility following synchronization of estrus with PGF2α in cows undergoing AI at detected estrus reported to be normal or above normal by various authors and this variation could be attributed to differences in the interval between treatment with PGF2α and ovulation. PGF2α is also capable of reducing the voluntary waiting period, thereby improving the overall reproductive efficiency. Administering PGF2α during the early postpartum hastens the uterine involution (Young et al. 1984). There exists a correlation between time to first postpartum ovulation with normal luteal phase and completion of uterine involution in cattle (Madej et al. 1984). However, the effect of PGF2α administration in the early postpartum period revealed no increase in pregnancy rate to first AI, but reduced the service period significantly (Burton and Lean 1995). Different protocols for estrus synchronization using PGF2α are shown in Fig. 9.8a–g.

9.5.1.3 Controlled Internal Drug Release (CIDR®) and PGF2 Alpha

The use of CIDR devices has increased recently in controlled breeding programs. For the manufacture of Controlled Internal Drug Release device, the silicon elastomer and progesterone powder (10% w/w) are mixed and molded around a nylon spine under a very high temperature of 190–220 °C. The CIDR is a "Y"-shaped thin flexible device and can easily be inserted intravaginally using the applicator by following sterile procedure. The device has a flexible nylon thread attached to the distal end long arm of the device, which hangs outside the vagina after insertion, indicating the retention of the device and also facilitates its easy removal after the desired period as per the chosen protocol. The quantity of progesterone loaded into CIDR device is 1.38 g and the device once it is kept inside the vagina, it should maintain an elevated plasma progesterone of at least 2 ng/ml for 7–10 days. The silicon-based progesterone-releasing devices when placed intravaginally allow continuous release of progesterone across the vaginal wall and get access to the systemic circulation. These devices are placed intravaginally for at least 7 days with a shot of PGF2 alpha a day before the removal of device, followed by detection of heat and AI in 48–96 h.

9.5.2 Progesterone-/ PG-/GnRH-Based Protocols

Pursley et al. (1995) from the University of Wisconsin-Madison, USA, developed a new protocol "Ovsynch" (ovulation synchronization) for synchronization of ovulation time using PGF2alpha and GnRH in dairy cows, which allows us to perform AI at a fixed time within the expected period of ovulation and also eliminates the need
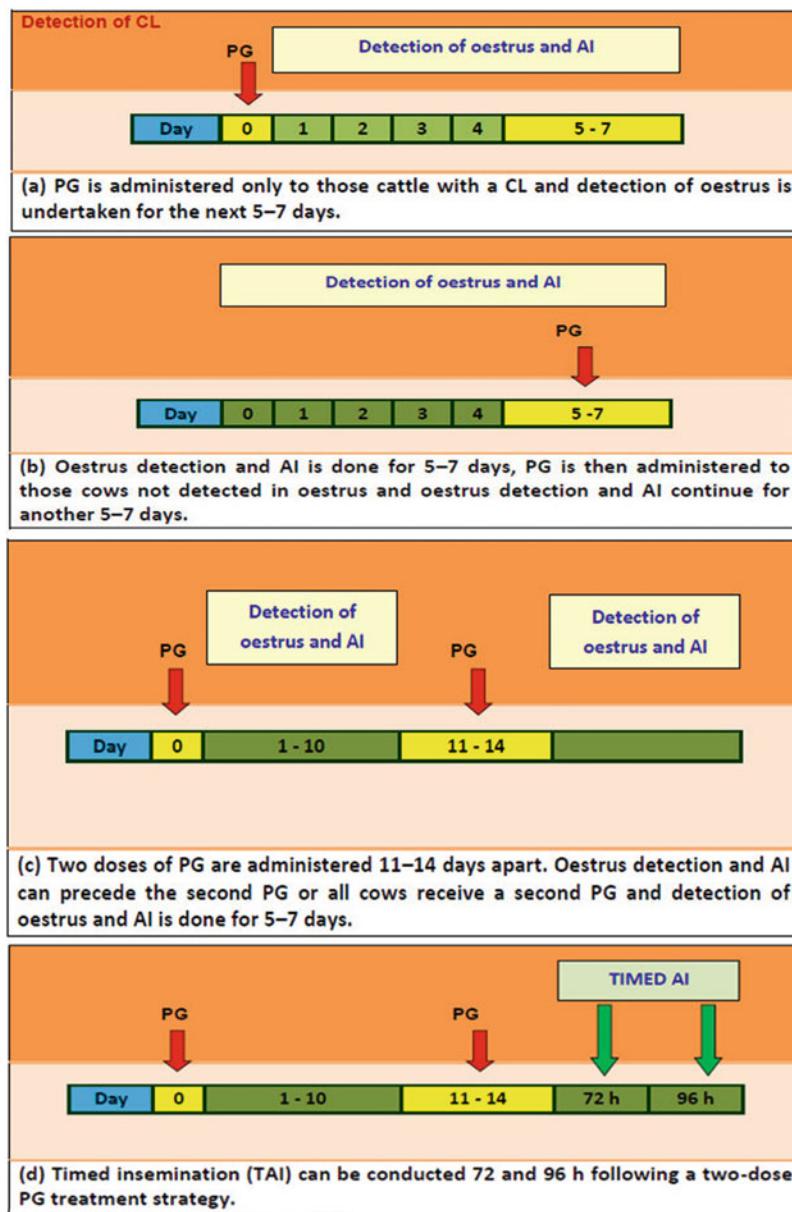
Fig. 9.8 (**a–g**) Prostaglandin-based protocols

for detection of estrus. In Ovsynch protocol, the initial GnRH injection of commercially available preparations induces the ovulation of the dominant follicle that forms a CL. Following ovulation, a new follicular wave emerges in 48 h. The PGF2 administered on day 7 of first GnRH injection induces luteolysis. With AI, the GnRH injection (100µg of gonadorelin or 10µg of Buserelin) is administered 48 h after the PGF2 that induces the ovulation of the dominant follicle in next 8–24 h.

Co-Synch protocol: In this protocol, the timed AI (TAI) is carried out at the time of the second GnRH injection and this reduces one less handling required for each

(e) Targeted breeding program: PG is administered at 14-day intervals with detection of oestrus and AI being done throughout the program. Timed insemination of cows not detected in oestrus can be conducted 72–80 h after the third dose of PG.

(f) Synchrony of oestrus can be improved by administering oestradiol benzoate (EB) 48 h after a two-dose PG treatment strategy.

g) Administration of EB and hCG 12 h after administering PG to cows with a CL can be used to synchronize ovulation and enable timed AI 36 h later.

Fig. 9.8 (continued)

cow compared to Ovsynch protocol. However, in comparison to TAI at 12–18 h in Ovsynch protocol, the conception rates may not be optimized.

Pre-Synch: In this protocol, two PGF2 alpha injections are given 12–14 days apart before the initiation of Ovsynch protocol to achieve better synchrony.

Heat-Synch: It is a modified approach to Ovsynch in which 1 mg (0.5 ml) of estradiol, a highly fat-soluble derivative with a prolonged estrogenic effect, is administered intramuscularly 24 h after PGF2 alpha injection instead of GnRH 48 h after PGF2 alpha injection to achieve ovulation.

Hybrid-Synch: This protocol is a combined approach of the Select Synch and Co-Synch. It involves the GnRH on day 1 and PGF2 alpha administration on day 8 followed by estrus detection and AI. The nonresponders are bred on day 11 with an injection of GnRH. Lower cost and less handling are the major advantages compared with Ovsynch and Co-Synch. The Hybrid-Synch protocol achieves highest conception rates among all other GnRH-based programs.

9.6 Management Considerations

Estrus synchronization programs require good management, regardless of the protocol used.

- Cattle must be maintained under proper nutritional and health status.
- Animal safety must be ensured while restraining, handling, and breeding.
- Estrus synchronization products being mostly of hormones, the pregnant women and persons with respiratory issues, such as asthmatics, should handle all these drugs with precautions (i.e., avoid direct contact with skin and other body parts while handling and administering) and follow the manufacturers guidelines strictly or better avoid handling.
- The hormones must be administered in proper dosage by proper technique for ensuring the success of the protocol. The drugs should be stored, used properly as per the directions of manufacturers and shelf-life limitations, and should be meticulously followed.

The estrus synchronization programs primarily aim at reducing the calving to conception interval. The protocols are being continuously evolved in order to achieve precise synchrony of ovulation in the whole dairy herds at predetermined times.

9.7   A Novel Nanoparticle-/Nanofiber-Based Controlled Progesterone-Releasing Approaches: A Way Forward for Estrous Synchronization in Bovines

The synchronization of estrus in cattle and other animals has been in practice through various synchronization protocols form the last 45 years since 1975 onwards especially, in large dairy herds in western countries. Later on, estrous synchronization protocol has been widely practiced all over the world in bovines: it offers several advantages through large-scale fixed-time artificial insemination without the need for estrus detection, to improve the rate of genetic progress by increased use of semen from superior sires and by reducing the generation interval (Mauleon 1974). Progesterone is one of the major steroidal hormones and it is central to synchronization of the estrus and ovulation cycles in bovine estrus synchronization protocol. Progesterone (P4) hormone was originally used to synchronize the bovine estrous cycle (Lamond 1964; Gordon 1976). In animals, especially in ruminants (Rathbone et al. 1998: Wheaton et al. 1993), progesterone (P4) delivery is mainly through P4-impregnated ear implant and intravaginal silicon elastomer inserts. Implants are invasive in nature; hence, their use is limited in veterinary treatment especially under field conditions due to lack of basic facilities for surgery, manpower, handling of large animal, chances of infection, and requirement of repeated use, which is very much stressful to animals. It has been reported that cattle supplemented with synthetic P4 melengestrol acetate (MGA) orally (Loiola et al. 2009) showed estrus expression, but preovulatory follicle diameter is not effective as predictors for selection and also disadvantages associated with oral feeding; hence, it is not a preferred choice for P4 delivery. Several attempts have been made to develop controlled release of P4 for achieving plasma progesterone concentrations above 1.5–2 ng/ml causing regression of dominant follicle and to suppress the pulse frequency of endogenous luteinizing hormone (LH) (Roche et al. 1981) for the period of 12–15 days. Plasma progesterone concentrations will fall below 1.5 ng/ml within 1 h after the removal of device. Then in 1993, Macmillan and Peterson devised a silicon elastomer–based intravaginal progesterone-releasing device for cattle (CIDR- B: 1.9 g progesterone with 100 mg oestradial benzoate) for estrous synchronization, and infertility management. Most of the veterinary progesterone delivery systems are made of synthetic nondegradable polymers such as silicone (Si), polyurethane (PU) and ethylene-vinyl acetate (EVA) copolymers, as they are biocompatible and inexpensive.

Development of alternative controlled P4 delivery platform/devices for estrus synchronization in bovines through the use of modern technological advances especially nanotechnology-based delivery platform for P4 delivery has been in progress. There are several biocompatible and biodegradable nanosized delivery systems (starch/cellulose/synthetic polymers like PLA/PGA/PLGA/PEG) alone or in blend form have been developed to improve their bioavailability through controlled release for several days to enhance the therapeutic efficacy of several bioactive molecules/drugs, (Kayaci and Uyar 2012: Oliveira et al. 2013; Maghsoudi et al. 2020). Nanofiber-based products are widely employed in various industries ranging

from food packaging/food preservation to several types of targeted drug delivery including controlled drug delivery in human medicine for various disease treatments (Gayatri and Bindu 2018). In humans, L-cysteine-based solid lipid nanoparticle–based intravaginal PR delivery has been studied by Cassano and Trombino in Cassano and Trombino 2019 and for preventing preterm delivery by nanofiber-based intravaginal progesterone-loaded patches by Cam et al. 2020. However, their potential use has not been harnessed in animal science.

Recently, several biocompatible and biodegradable nanosized delivery systems have been developed to enhance the therapeutic efficacy of active pharmaceutical ingredients, by improving their (Kayaci and Uyar 2012: Maghsoudi et al. 2020). The advantages of using nanofiber-/nanoparticle-based biomolecule delivery system is increased surface area, reduced dosage, and efficient release, which may avoid repeated handling stress and frequent hormone administration. Moreover, P4 release in CIDR is through surface erosion, and only 1 mm thickness of CIDR surface will undergo erosion. 1 g of residual P4 has been estimated in CIDR after single use due to the covalent bonding in silicone matrixes, which is an intrinsic attribute of these systems (Rathbone et al. 2002: de Graaff and Grimard 2017). Also, PRID and CIDR silicon elastomer devices are loaded with high doses of P4, that is, 1.55 g and 1.38 g, respectively, but these disadvantages can be efficiently addressed in nanoparticle- or nanofiber-based P4 hormonal delivery.

A nanosized delivery method offers an efficient drug/hormonal delivery at lower dose, thereby avoiding adverse systemic affects. It requires proper understanding of physiochemical properties and functional groups precursor of nanopolymers, that is, polymer and biomolecule to be delivered. Intravaginal delivery can provide controlled release of hormone avoiding extensive systemic metabolism. Moreover, off-target side effects and sudden progesterone metabolism through hepatic circulation can potentially be avoided. Considering the several advantages of intravaginal drug delivery, CIDR for progesterone has been in use in cow, sheep, goat, and swine since 1980s (Rathbone et al. 1998). In 2002, Rathbone et al. demonstrated that injection molded biodegradable polyester poly (ε-caprolactone) containing 10% (w/v) progesterone can be used for controlled delivery of progesterone via vagina in cattle, which is clinically effective as commercially available CIDR device. Electrospinning method is commonly employed for production of nanofibers, which offer several advantages such as an extremely high surface-to-volume ratio, tunable porosity, malleability to conform to a wide variety of sizes and shapes and the ability to control the nanofiber composition, to achieve the desired results from polymer properties, functionality and electrospinning processing parameters (Akhgari et al. 2017: Sunil et al. 2015: Haider et al. 2018) are exploited to achieve immediate drug release. A wide variety of electrospun nanofiber polymers have been customized for prolonged controlled release of drugs from antibiotics to cancer drug delivery (Kajdič et al. 2019). In Karuppannan et al. 2017, Karupannan et al. used electrospinning method for production of progesterone-loaded zein-based biodegradable nanofibers for progesterone delivery. Based on the in vitro studies, the authors suggested that 1.2 g of progesterone-loaded zein nanofibers can be potentially used for estrus synchronization in livestock animals through controlled

delivery of progesterone. Helbling et al. in Helbling et al. 2019 designed reusable intravaginal device made from ethylene vinyl acetate (EVA) for estrus synchronization in cattle, and concluded that concentration values higher than 2 ng/ml can be achieved over a period of 7 days by using EVA inserts and can be used for estrus synchronization of nonlactating beef cattle at farm level. The possibility of generating progesterone-loaded mucoadhesive fibers from a blend of polymers to understand mucoadhesive interactions between progesterone-loaded fibers (with varying carboxymethyl cellulose (CMC) content) and mucin has been studied for drug delivery (Brako et al. 2018a, 2018b). In 2020, Cam et al. reported that engineered intravaginal P4-loaded poly (lactic) acid (PLA) fibrous patches produced from electrospinning and pressurized gyration techniques showed controlled P4 release in 15–22-day pregnant mice, which was equivalent to third trimester of human pregnancy over a period of 7 days with a similar profile.

In summary, nanoparticle-/nanofiber-based P4 delivery has a huge potential in animal science and much of it is in nascent stage. Hence, more research on nanoparticle-/nanofiber-based P4 delivery has to be initiated to make use of the advantages associated with nanotechnology and for the development of an alternative low-cost eco-friendly nanotechnology-based polymeric nanoparticle-/nanofiber-based P4 delivery platform/device in bovines for estrous synchronization (ES) and for pregnancy to avoid early embryonic mortality.

9.8   Aromatase Inhibitor for Estrous Synchronization and Fixed Time Ai in Cattle

There is a need for development of cost-effective controlled breeding program protocols with fewer animal handlings. Andersson and Skakkebaek (1999) stated that increasing consumer sensitivity to the possible deleterious effects steroid-based synchronization protocols especially of estrogens in food and in the environment has resulted in development of new regulations about the use of estrogenic products in livestock.

Through negative feedback mechanism on the hypothalamo-pituitary axis gonadotropin release is suppressed by oestradiol hormone. The recruitment of a new wave of follicular development occurs due to a new surge of FSH when suppressive effects of oestradiol are removed by administering aromatase inhibitors (Requena et al. 2008). Verpoest et al. (2006) stated that aromatase inhibitors are used in humans with Polycystic ovarian syndrome for inducing normal folliculogenesis, ovarian stimulation for oocyte collection and IVF, and for fertility preservation in women undergoing cancer therapies.

Yapura et al. (2017) have reviewed extensively and explained that based on the chemical structure and mechanism of action, there are Type I or Type II aromatase inhibitors. Compounds derived from androstenedione are classified as Type I steroidal aromatase inhibitors. These aromatase inhibitors cause lasting and selective inhibition due to its irreversible binding to the active site of P450arom, which would induce a covalent change on the structure of the enzyme. The Type I

inhibitors viz. Formestane and Exemestane (Goss and Strasser 2001) are called as "suicide inactivators" because of their irreversible effects. The triazole groups with a nitrogen-containing heterocyclic moiety as a common characteristic of their chemical structure are classified as nonsteroidal aromatase inhibitors (Type II). The Type II estrogen synthetase-specific inhibitors viz. Letrozole, Anastrozole, and Fradozole bind to the heme group and occupy part of the active binding site of the enzyme in the P450arom and interfere with enzyme activity in a reversible manner. Letrozole treatment leads to extension of lifespan of the dominant follicle and thereby delayed emergence of the next follicle wave and/or ovulation. In human infertility patients, Letrozole is used for ovulation induction. In addition, Letrozole administration in heifers showed a larger CL size and/or higher circulating concentrations of progesterone indicating its luteotrophic property.

Yapura (2012) observed that the transient elevation of estrogen concentration 12 h after sponge insertion intravaginally together with inhibin results in arresting progress of follicular wave at stage of dominance showing a picture of overdominance (prolonged growth and maintenance of dominant follicles). Based on an another study, Yapura et al. (2016) concluded that in heifers, higher synchrony of ovulation and ovulation rate was obtained when letrozole-impregnated intravaginal device was kept for 4 days followed by PGF2α treatment at device removal, and GnRH 24 h. Further, it is opined that in bovines and in ewes, Letrozole-based protocol may be adopted at random stages of the estrous cycle and this shows that Letrazole-based protocol can be used for estrous synchronization (Abdel Dayem et al. 2020).

9.9 Estrous Synchronization in Zebu (*Bos indicus*) Cattle and Buffaloes (*Bubalus bubalis*)

Reproductive physiology and performance differ between *Bos taurus* and *Bos indicus* cattle. The zebu cattle show several anatomical/morphological and functional differences in their reproductive system from *Bos taurus* including variations in circulating hormones and metabolites. The ovaries of Zebu cows are smaller than taurine cows and it was reported that the CL was embedded deep within ovarian stroma. The duration of estrus was shorter and mostly occurred during night hours in Zebu cows with the smaller size of preovulatory follicle and corpus luteum. (Gordon 2004). Viana et al. (2003) observed that the maximum diameter of the follicles was lesser and dominant follicles showed persistency in Zebu cattle of Brazil. The studies in the field of reproductive biology in cattle have mostly involved in *B. taurus* breeds of temperate regions. Further, the reproductive management strategies/technologies including synchronization protocols, which have been used in *Bos taurus* cattle have been exploited as such or with slight modifications may not be ideal for *B. indicus* cattle of tropical regions. Recently, estrous synchronization, fixed time AI, and ART protocols are being optimized based on the acquired knowledge in *Bos indicus* breeds. In literature, most of the studies on *Bos indicus* breeds are available for Nellore cows and to some extent in Ongole and Gir cattle. The Indian subcontinent is

bestowed with 50 recognized cattle breeds belonging to milch, draft, dual purpose and dwarf breeds and only meagre information is available on their reproductive physiology and uniqueness. Considering the breed-specific variations, there is an urgent need for exploiting our understanding the reproductive function with modern scientific approach and tools especially the ovarian physiology to better adopt the controlled breeding methods and advance reproductive technologies for the genetic improvement, faster multiplication of germplasm, and conservation.

Pinheiro et al. (1998) observed that in nulliparous Nellore heifer after PGF2-α-induced and natural estrus, the time duration between the onset of estrus and ovulation was 27.7 ± 2.4 and 26.1 ± 1.2 h, respectively. Besides, in PGF2α-induced and natural estrus, the estrous behavior, was lesser compared with *Bos taurus* breeds, and estrus was observed mostly during night hours, which impaired AI coverage. Ultrasonographic observation after PGF2α-induced cycle revealed that the majority of the Nellore heifers ovulated within 7 days post PG treatment and surprisingly 50% of them were not observed to be in standing heat. Additionally, a higher proportion of *Bos indicus* (Nellore) females did not respond to PGF2α alpha even after administration of regular dosages and this needs to be studied by further studies. This study clearly showed the variations in estrus behavior and ovulation time and luteal function in Nellore (*Bos indicus*) breed.

Indigenous breeds of India adapt well to hot, humid climates and diseases of tropics worldwide (Gaur et al. 2003). But they exhibit delayed onset of puberty, have extended postpartum anestrus periods, and are slow breeders when compared to exotic breeds (Singh and Khurana 1998). In indigenous crossbred cattle, anestrus and repeat breeding constitute as a major cause of infertility (Kutty et al. 2003). Various hormonal preparations and protocols have been used to induce onset of puberty, thereby reducing the age at first calving and induce cyclicity in anestrus (Dhami et al. 2015; Masoumi et al. 2017) cattle. Archbald et al. (1994) reported that estrus response following an injection of PGF2α was 55% within the period of 7 days in lactating cows. Galina and Arthur (1990) reported that despite palpable corpus luteum present in *Bos indicus*, the estrus exhibited after synchronization with PGF2α was less (30%) compared to 90% response in *Bos taurus* cattle. Alonso et al. (1995) reported that 80 to 100% of Zebu cattle showed regression of CL after synchronization with PGF2α. Out of these, PGF2α response cows only 29–60% cows exhibited the oestrus response and only 51% of the cows ovulated during the span of 5 days after treatment. Sahatpure and Patil (2008) have reported that two shot prostaglandin in nondescript and crossbred cattle in India showed 80% and 100% estrus rates, respectively. Sprott and Carpenter (2007) and Paul et al. (2015) observed that the estrus expression after synchronization with PGF2α showed over 2 to 8 days depending upon the stage of the cycle. Similarly, Jemal et al. (2020) reported the administration of single injection of PGF2α in indigenous Boran and crossbred heifers resulted in induction of estrus within 2–5 days after treatment. Studies on Deoni (Bos indicus) cattle breed of South India by Farooq and Jeyakumar (2021) observed that at the time of PGF2α injection (day 0), the mean diameter of the dominant follicle was 7.28 ± 1.30 mm. The diameter of the DF increased from the day of treatment to ovulation. The mean diameter of the DF in Deoni cows was

8.16 ± 1.15 mm and 9.73 ± 1.21 mm on day 1 and day 2, respectively. The diameter of the preovulatory follicle was 11.85 ± 0.93 and was observed on day 3–4 after PG treatment. The diameter of dominant follicle differed significantly on day 0 and 2 after treatment. However, the difference in DF diameter between day 0 and day 3 after treatment was highly significant ($P = 0.003$).

Estrus synchronization regimes including Ovsynch, Heatsynch, and exogenous progesterone devices like CIDR, PRID, and Norgestomet ear implant are used to improve conception rate in indigenous dairy bovines with less number of services per conception, thereby achieving ideal intercalving interval of 12–13 months (Chaudhari et al. 2012; Nak et al. 2011). In Kankrej anestrus cows, Heatsynch, Ovsynch, and Ovsynch + CIDR estrus synchronization protocols induce estrus and Ovsynch + CIDR protocol provides a better conception rate and fertility. Doublesynch and Estradoublesynch protocols efficiently induced estrus in Gir prepubertal heifers with improved conception rates (Chaudhary et al. 2018). Estrus synchronization in Ongole cows with GnRH and PGF2 provides better synchrony and remarkably decreased the service period and intercalving period. However, double injection of prostaglandin yielded a better conception rate than the Ovsynch protocol (Ramana et al. 2013). The Ovsynch and PGF2α induced estrus response and ovulation effectively and resulted in about 43% and 31% conception rate, respectively, in Sahiwal cows.

Deshmukh et al. (2010) showed that in Red Kandhari cows, the Selectsynch protocol resulted in induction of estrus in 66.67% with a conception and pregnancy rate of 87.50 and 58.33%, respectively, which is comparatively lesser than Ovsynch protocol. Overall, Ovsynch and endogenous progesterone device–based protocol are more effective in inducing estrus in prepubertal and postpartum animals, thereby augmenting fertility in indigenous cattle. The evaluation of seasonal influence in response to Ovsynch, Heatsynch, and PRID protocols in true anoestrus cows revealed a better estrus induction response of 100% in the winter season compared to 80–90% in the summer season. In the summer season, the conception rates were 20–30% compared to 40–60%, in the winter season, indicating that winter is more suitable for implementing estrus synchronization regimes in indigenous cattle (Borakhatariya et al. 2017).

9.10 Estrous Synchronization in Buffaloes

9.10.1 Reproductive Physiology

In buffaloes, the estrous cycle duration is similar to cattle and ranges between 17 and 26 days with a duration of estrus between 5 and 27 h. The ovulation occurs about 24–48 h (mean 34 h) after onset of estrus, or 6–21 h (mean 14 h) after the end of estrus. During summer or hot season, the estrus duration used to be shorter and estrus is usually expressed in the night or early morning hours. Buffalo heifers exhibit a prevalence of two-wave cycles in comparison to adult cows, which shows mostly two or three follicular waves in a cycle. Buffaloes with two follicular waves show

shorter duration than three-wave cycles (21 vs 24 days). The DF size of the first wave is similar to the second wave: approximately 15 mm. Three wave of follicular growth showed a longer luteal phase, interovulatory interval, and estrous cycle duration.

9.10.2 Methods to Synchronize Estrus

Estrus and ovulation synchronization protocols generally involve controlling the luteal function, that is, induction of luteolysis using prostaglandins or extending the luteal phase using progestational compounds. However, to accomplish enhanced synchrony of ovulation and improve fertility, a more defined manipulation of follicular development is considered necessary by the use of various combinations of hormones viz. GnRH, FSH, LH, eCG, hCG, prostaglandins, progesterone, and oestradiol.

1. Administration of prostaglandins or progesterone analogues results in controlling the luteal phase of the cycle.
2. Use of different combinations of prostaglandins, progesterone, GnRH, hCG, eCG, and oestradiol results in more or less precise control of follicle development and ovulation.

9.10.3 Manipulating the Luteal Phase of the Cycle

9.10.3.1 Prostaglandin (PG) Protocols
The luteolytic effect of PGF2 alpha is similar to cattle and causes luteolysis of corpus luteum from day 5 of cycle and leads to sharp reduction in the levels of progesterone followed by estrus and ovulation. PGF2 alpha can also be administered through intra-vulvo submucosal route (Fig. 9.9a–f) ipsilateral to CL.

9.10.3.1.1 PG Injection Regimen

Regimen–I
Prior to treatment, animals should be examined rectally to detect whether they are anatomically normal, nonpregnant, and have a mature CL. Estrus is expected to occur 2–5 days following PG injection; during this time, they are inseminated. Treated animals should be inseminated at the usual time relative to detection of estrus. If estrus detection is not desirable or possible, treated animals may be inseminated either once at about 72 h post injection or twice at about 72 and 96 h. In this case, the double insemination is advised for better conception rates.

Regimen–II
The cyclicity status should be assessed before treatment with single PG injection regimen accomplished by heat detecting and breeding at the usual time relative to
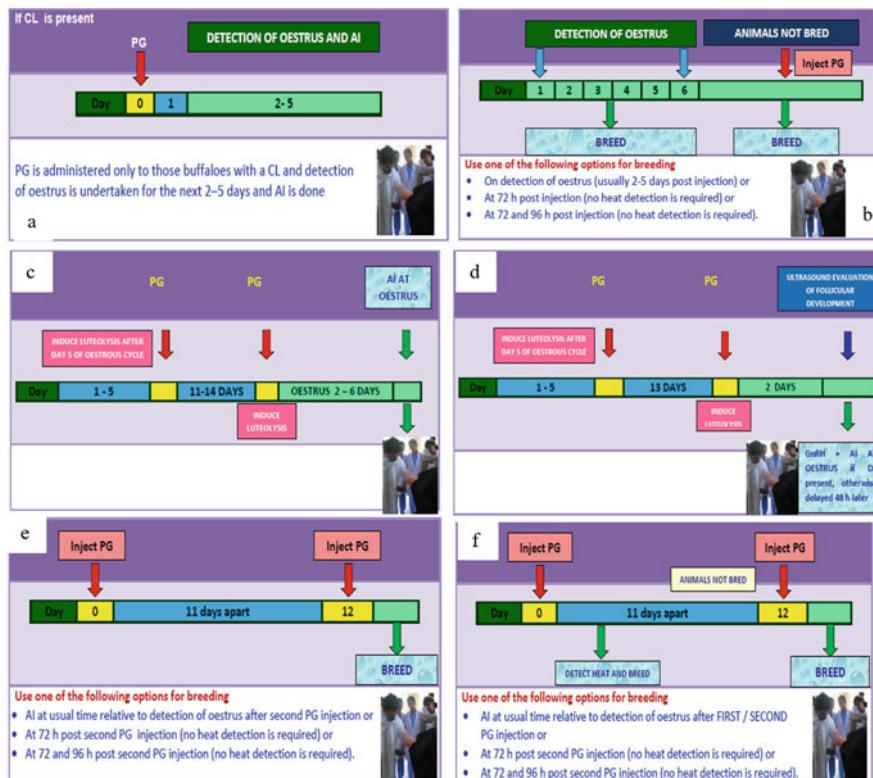
Fig. 9.9 (**a–b**) Prostaglandin based synchronization protocol. (**c–f**) Prostaglandin based synchronization protocol

estrus for 6 day period prior to injection. Animals detected are inseminated or bred naturally in the usual manner. If by the sixth day, the cyclicity status appears normal, all animals not already inseminated should be palpated for normalcy, nonpregnancy, and cyclicity and, if normal, injected with PG. Breeding should then be continued at the usual time relative to any occurrence of estrus on the seventh and eighth days. On the ninth and tenth days, breeding may be continued at the usual time relative to estrus or all animals not already inseminated may be bred either once on the ninth day (72 h post PG injection) or on both the ninth and tenth days (at about 72 and 96 h post PG injection).

9.10.3.1.2 Double PG Injection Regimen

Regimen–III

Prior to treatment, animals should be examined rectally to detect whether they are anatomically normal, nonpregnant, and cycling. When the first injection of a double injection regimen is followed, the presence of a mature CL is not necessary. A

second injection should be administered 11 days apart from the first PG injection. Estrus is expected to occur 2–5 days after second PG injection in normal cycling animals.

Regimen–IV

After first PG injection, many animals will come in to estrus. These animals can be inseminated at the usual time relative to estrus. Animals not observed in estrus should receive a second injection 11 days apart from the first PG injection.

9.10.4 Progesterone Protocols

In buffalo, use of progesterone or progestagens (mainly CIDR, PRID or CRESTAR) are limited to synchronize estrus and ovulation. These protocols additionally include the administration of estradiol valerate (or benzoate) at the time of progestagen application, and/or PGF2alpha on the day before progestagen removal. Estrus is observed in 80–93% and ovulation occurs 40–96 h following progestagen withdrawal. Collectively, progestagen treatments during the breeding season result in satisfactory conception rates. Hiremath and Ramesha (2015) suggested that in buffaloes with poor estrus symptoms/anestrous were treated with CIDR device along with Cidirol treatment is beneficial in buffaloes diagnosed with true anoestrus and.

9.10.5 Based on Controlling the Follicle Development and Ovulation

9.10.5.1 GnRH-PG Treatments

In buffaloes, GnRH administration induces ovulation in 60–86% of treated animals with occurrence of ovulation post GnRH administration was 33 ± 8.3 h. AI may be carried out between 12 and 24 h after the last GnRH administration of the Ovsynch protocol. The Ovsynch protocol resulted in synchronized ovulation in 78–90% of animals, with the conception rate between 33% and 60%. Artificial insemination after heat detection or TAI yields similar pregnancy rates. (Fig. 9.10).

9.10.6 Protocols for Low or Nonbreeding Season

The seasonal anestrus and reduced reproductive activity is associated to climatological and local weather factors such as ambient temperature, relative humidity, day length, and rainfall. Summer anestrus is characterized by reduction in the circulating levels of pituitary and gonadal hormones. Various factors or vents have been associated viz. higher levels of prolactin, reduced levels of FSH and LH secretion or peaks due to suboptimal functioning of HPA axis and variable circulating progesterone plasma concentration. Postpartum ovarian activity is significantly delayed compared to the breeding season (38–64 to 116–148 days) during seasonal
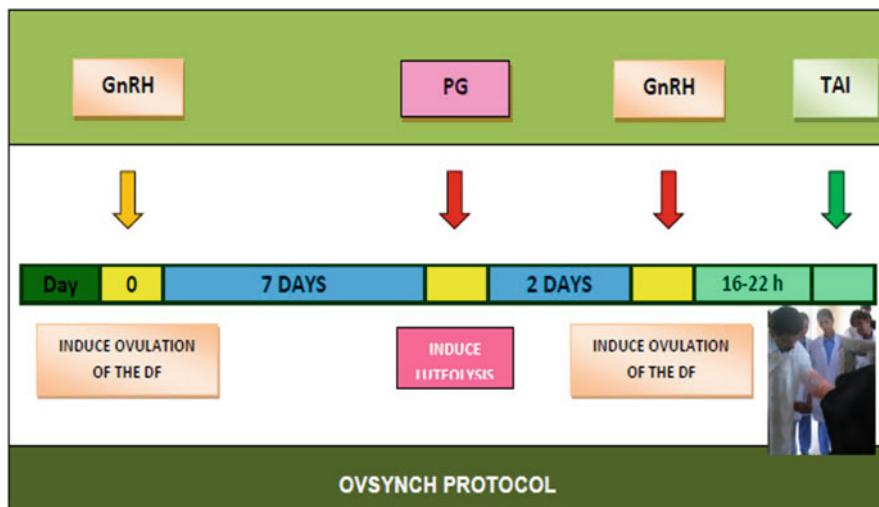
Fig. 9.10 Ovsynch protocol

anestrus with increase in interval from parturition to first estrus. The duration of estrus also is reduced to 8–10 h during summer season. In summer, about 30% of cyclic animals show no estrus behavior; however, about 64–75% buffaloes in low breeding season exhibit variable degrees of ovarian activity.

9.10.6.1 Protocol

Administration of CIDR for 7 days between the first GnRH administration and PGF2alpha injection improved the fertility during low-breeding season. In anestrous buffalo cows, the resumption of estrus could be achieved with progestagens in conjunction with eCG with a pregnancy rates close to 30%. The use of progesterone-based protocols allows the insemination and induction of pregnancy in nonproductive animals during the nonbreeding season (Fig. 9.11).

9.10.7 Outcomes of Various Synchronization Protocols in Buffaloes (*Bubalus bubalis*)

Effective use of artificial insemination (AI) in buffaloes is hampered by various factors like 30–40% reduction in estrus detection rate, duration of estrus, and prediction of time of ovulation. To address these issues, in recent years, to manipulate follicle growth dynamics, many controlled breeding protocols have been developed to precisely control ovulation synchrony to improve reproductive performance (De Rensis and Lopez-Gatius 2007). During last six to seven decades, the fertility following estrus synchronization protocols using PGF2 alpha or using progestogens varied widely (Ahmad and Arshad 2020).
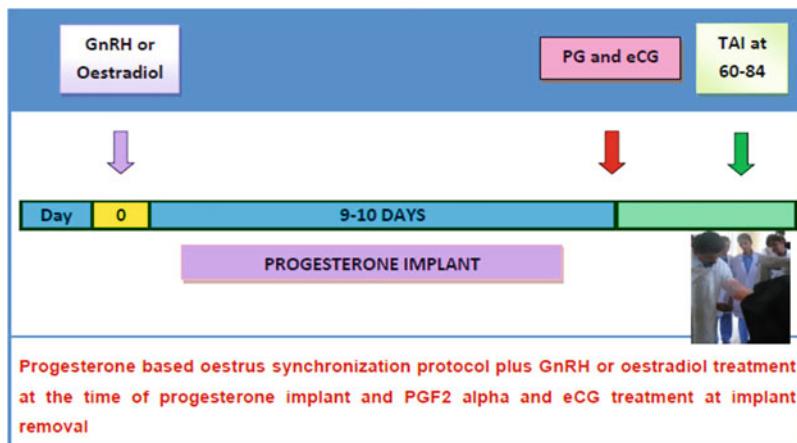
Fig. 9.11 CIDR + Ovsynch protocol

The PGF2α injection given around day 5 of the cycle resulted in lysis of the CL with subsequent reduction in plasma P4 levels and induction of estrus and ovulation within 24 h. (Chaudhary et al. 2015) and similar response was obtained when PGF2 alpha was administered through intra-vulvo submucosal route (Chhatry et al. 1999; Chohan 1998). The time interval from PGF2alpha to estrus occurred at 88 h with an average pregnancy rates of 45–50 per cent. However, the conception rate falls below 25% even though more than 80 per cent of buffaloes showed standing estrus. (Chaudhary et al. 2015; Cochran et al. 1998; Chohan 1998; Crudeli and de la Sota 2011; Dadarwal et al. 2013; Day and Geary 2005; De Santis et al. 2003).

In buffalo, the use of CIDR, PRID, or CRESTAR for synchronization is limited and CIDR and PRID, which are available for cattle, are widely used in buffaloes. The implants are applied for 8–10 days (Ghuman et al. 2009; Murugavel et al. 2009). Withdrawal of progestagen implant resulted in estrus and ovulation within 48–96 h (Bhosrekar et al. 1994). In progestagen-based protocols, the estrus and conception rate were improved by the administration of estradiol, hCG, and/or prostaglandin (Bachlaus 1980). Barile et al. (2001) reported that administration of eCG at the time of progesterone device withdrawal followed by two timed artificial inseminations at 72 and 96 h resulted in conception rate of 51%.

Dadarwal et al. (2013) reported that administration of GnRH causes ovulation in 33 ± 8.3 h in 60–86% of treated buffaloes. The use of Ovsynch protocol resulted in synchronized ovulation of 78% and 90% of animals, with conception rates of 33%, 50%, and 60%. It was observed that 87.5% of heifers and 100% of adult buffaloes ovulated when the size of the largest follicle was >8 mm at the time of first GnRH injection (Derar et al. 2012). Purohit et al. (2019) reported that the estrus and pregnancy rate on the use of Ovsynch protocol ranged between 41.6 and 91.9 and 1.11 and 68.8%, respectively.

In Heat synch protocol, the second GnRH is replaced with estradiol, which resulted in estrus induction and conception rates of 100 and 50%, respectively (Ali

et al. 2012). This protocol is considered to show better estrus expression; however, the conception rates were low (Akhtar et al. 2013), particularly in lactating buffaloes (Berber et al. 2005). The protocol has been suggested to be used with caution considering the potential dangers of milk suppression with estradiol in subestrus buffaloes for timed inseminations (Mohan et al. 2009; Carvalho et al. 2012b). Variable conception rates ranging from 18% to 60% were reported in Ovsynch protocol (Malik et al. 2011; Derar et al. 2012). Hoque et al. (2014) reported that presynchronization with PG 12 days before Ovsynch treatment resulted in slight improvement in pregnancy rates. Mirmahmoudi et al. (2014) achieved a pregnancy rate of 62–64% in an another protocol "estra-doublesynch" with PG on Day 0, GnRH on Day 2, PG on Day 9 and estradiol benzoate on Day 10 and timed insemination 48 and 60 h after estradiol benzoate was suggested for cyclic and anestrus Murrah buffaloes.

9.10.8 Resynchronization Programs

Ahmad and Arshad (2020), in a recent review, stated that the resynchronization program has been used as an intensive reproductive management option to cover all cows at the start of breeding season. Resynchronization with GnRH on d 23 post AI during post breeding season in buffaloes resulted in pregnancy per AI in treated and control group was 63% versus 41% with a cumulative pregnancy rate of 59% versus 81%, respectively. Similarly, in resynchronized program, there was decrease in late embryonic losses as compared to control group buffaloes (Ahmad et al. 2017). Neglia et al. (2018) reported that during low breeding season in nulliparous buffaloes, resynchronization with P4-based protocol resulted in 89.3% overall herd fertility.

9.11   CO-Synch with CIDR and FTAI Protocol in Prepubertal Heifers and Postpartum *Bos indicus*

9.11.1 (Deoni) Cattle

In Deoni cows, prolonged days from calving to first service, first to successful service, and service period significantly lead to higher calving interval. Therefore, there is a need to explore various strategies to reduce intercalving period to improve reproductive efficiency of Deoni cows.

The efficiency of Ovosynch + CIDR protocol (GnRH on day 0, CIDR inserted intravaginally for 8 days with PGF2α on day 7, and timed artificial insemination at 48 and 72 h after PGF2α and again GnRH on day 9) evaluated in postpartum Deoni cows resulted in 100% estrus induction rate and 60% conception rate as confirmed by progesterone assay on day 30. Therefore, it can be inferred that Ovosynch + CIDR protocol could serve as a valuable technology for improving reproductive efficiency postpartum Deoni cows (Jeyakumar et al. 2014) (Fig. 9.12).

Fig. 9.12 Induction of estrus using CO-Synch with CIDR and FTAI protocol and resultant synchronized calving Deoni (*Bos indicus*) cattle

9.12　Application of Real-Time Ultrasound Technology in Controlled Breeding Programs

Statement of O.J. Ginther explains the fact that, in large animals, the grey-scale diagnostic ultrasonography is the most profound technological advance in the field of basic and clinical reproduction (Ginther 1986). It is evident from exponential publications (research articles and books) in the recent decades that many discoveries, our understanding, and developed concepts especially in large animal reproductive (ovarian, uterine, and fetal) functions would not have been possible without the advancement in the field of reproductive real-time ultrasound technology, which further supports the statement of O.J. Ginther, a pioneer in the field of large animal reproductive ultrasonography.

Evaluation of ovarian structures viz. ovarian diameter, stroma, blood supply, follicles, cysts, and corpus luteum has become possible with real-time ultrasonography. The most unique observable structures of ovarian ultrasonography are the ovarian antral follicles, which are displayed as fluid-filled structures anechoic/nonechogenic black spherical structures of varying sizes, depending on the stage of follicular development. The other structures like the ovarian stroma, corpora haemorrhagica, corpora lutea, corpus albicans containing varying degrees of dense

cells appear as gray (hypo/hyper/mixed echoic) on the monitor. The growth and atresia of the individual antral follicles can be mapped and assessed by consecutive ovarian ultrasonography in bovines as ovarian follicular development occurs in a wave-like pattern with a common occurrence of two or three waves per cycle (4 or five waves per cycle have also been reported). Sequential ultrasonography is used to identify the growth stage of follicles (growing phase, static phase, and regressing phase) and corpus luteum [early developmental (CH), mid (mature CL) luteal, and late luteal (regressing) phase]. Ultrasonographically classified follicles and corpus luteum stages are assessed by analyzing the echotexture (pixel heterogenicity) of basic/high-resolution images and with an aid of computer-assisted image processing and was shown to correlate with functional attributes like follicular health and hormonal status. Though follicles above 5 mm diameter can be identified using ultrasonography, identification of follicles between 10 and 15 mm diameter via ultrasonography was found to be more sensitive than rectal palpation (Aslan et al. 2000). The ultrasonographic appearance of luteal structures varies with the stage of development. Corpora hemorrhagica, which is present preceding ovulation, are less echogenic and difficult to distinguish from ovarian stroma. The mature CL is easily identifiable as a spherical structure of mixed/less echogenic nature (typically referred to as "salt and pepper" appearance). Corpora lutea with central cavity/lumen or lacunae are easily appreciable as spherical anechoic spaces and are surrounded by a rim of luteal tissues, which can well be differentiated from follicle. The gray-scale sonographic appearance and color flow Doppler ultrasonography of CL may be used to assess the functional status and estimate the stage of estrous cycle (Pierson and Ginther 1984; Cochran et al. 1998). Recently, it has been demonstrated that the color Doppler ultrasound is an important tool for the detection of functional regression of CL than conventional CL morphology, echogenicity, or estimation of progesterone. Studies based on ultrasonography have revealed that functional regression of CL occurs earlier at about 24 h before structural regression after induced luteolysis by PGF2α (Schams and Berisha 2004; Siqueira et al. 2019). A strong positive correlation exists between luteal blood flow and diameter of corpus luteum (De Tarso et al. 2018). Siqueira et al. (2019) reported that CL blood flow gives an early prediction about functional luteal regression than morphological characteristics, echogenicity, and progesterone concentration. The accurate interpretations regarding CL function can be made by ultrasonographic examination in comparison to relying mainly on structural characteristics (Siqueira et al. 2019) The real-time functional anatomy of the bovine ovary can be visualized by transrectal B mode and color Doppler ultrasonography, which when combined with estrus synchronization programs can effectively improve the efficiency and success of the protocol. In implementing estrous synchronization programs in large dairy herds, the transrectal ultrasonography can be used as a quick cost-effective management tool to classify the animals into either luteal or follicular phase for implementing the right estrus synchronization protocol at the appropriate time. The estrous synchronization programs ultimately aim at reducing the intercalving period by manipulation of follicular waves and ovulation to facilitate fixed time artificial insemination without the need for labor-intensive estrus detection. The various drugs like GnRH, progesterone, estrogen,

PGF2α, etc., intended for this purpose can have roles limited to a particular phase of the estrous cycle or both follicular and luteal phases based on the active structures present on the ovary. The GnRH is used to induce ovulation of the dominant follicle and to achieve synchrony by recruitment of a new follicular wave, whereas the PGF2α can potentially induce luteolysis in the presence of active CL only.

The induction of estrous employing PGF2α-based synchronization protocol is mainly dependent on the presence of functional corpus luteum on ovary. This identification of the active CL on ovaries is largely based on the per-rectal palpation of the CL, but there is large variation in identification of a cow with CL suitable for the PGF2α treatment based on the skill of palpator. Ott et al. (1986) reported that the per-rectal palpation of CL by experienced palpator was only 77% reliable than progesterone concentration for identification of CL. Similarly, Archbald et al. (1993) had reported the relation of 79% between per-rectal palpation and progesterone concentration. The traditional identification of mature CL based on per-rectal palpation is obsolete method to identify the cows suitable for PGF2α-based synchronization program. Ribadu et al. (1994) reported that the identification of CL based on ultrasonography possesses high accuracy of 95% for identification of cows suitable for PGF2α-based synchronization program.

The optimum size of the preovulatory follicle during various estrous synchronization is a main factor that has profound effect on fertility and pregnancy rate (Dadarwal et al. 2013; Wiltbank and Pursley 2014). Based on ultrasonography, a large range of the ovulatory follicles differing in diameter (<9–17 mm) exists during estrous synchronization protocols in indigenous cattle (Meneghetti et al. 2009; Sá Filho et al. 2010). De Tarso et al. (2018)) reported that the maximum diameter of the POF and follicle blood flow was highest for beef cows than heifers during estrus synchronization as observed by color Doppler ultrasonography. Perry et al. (2007) observed the low pregnancy rate in cattle induced to ovulate smaller sized follicles during GnRH-based estrous synchronization as observed by ultrasonography. However, such difference was not observed during natural induction of ovulation. Siddiqui et al. (2009) reported that those cattle having follicles with largest diameter and highest follicular wall blood flow during estrus had the maximum probability of becoming pregnant during synchronization protocols.

Identification of cattle failed to conceive after TAI receives greater significance. The nonpregnant cows identified earlier can be subjected to alternative management strategy including resynchronization of ovulation by which the days open can be effectively reduced. The use of ultrasonography can help the implementation of resynch protocols earlier by day 26 post insemination compared to day 33 transrectal palpation (Fricke et al. 2003). The accurate early identification of nonpregnant cows using ultrasonography has a positive effect on reproductive efficiency by reducing the days open and greater economic net return (Giordano et al. 2013). Rechecking of pregnant animals again is also critical when early pregnancy diagnosis is carried out using ultrasound as the embryonic loss at a later stage can cause potential economic loss.

The sensitivity and specificity of the detection of functional ovarian structures and pregnancy is far greater through transrectal ultrasonography, its usage in the

resynchronization programs can more than compensate for the low estrus detection rates (Pieterse et al. 1990; Shephard and Morton 2018). The cows without a CL at the initiation of resynchronization program with Ovsynch have poor conception rates, which on detection with transrectal ultrasonography can provide an alternative management opportunity for estrus induction. The presence of functional CL is of paramount importance for successful synchronization with PGF2α. Selective administration of PGF2α to animals with a threshold CL diameter namely the ultrasynch program had a short time to conception compared to the traditional Ovsynch program (Bicalho et al. 2008). The combined use of ultrasound for detection of functional CL and selective submission of animals to PGF2α coupled with a good estrus detection rate can be an economical and valuable alternative synchronization program (McArt et al. 2010). Ronci and De Rensis in an unpublished report mentioned about the use of ultrasonography to monitor the follicular growth in synchronization program and suggested that monitoring ovarian activity by ultrasound 2 days after the second PGF2alpha administration showed the size of dominant follicle at the time AI at 16–22 h later was at least 10 mm.

The reproductive ultrasonography can serve as an effective tool to detect the response to different estrous synchronization regimes. The percentage of cattle that fails to ovulate during estrus synchronization and TAI protocols is one of the major contributors to the reduced success rate (Stevenson et al. 2003). Incomplete luteolysis reduces the efficiency of GnRH-based regimens leading to the formation of persistent follicles and more variation in the estrus exhibition and ovulation rates. The proportion of cows ovulating and developing a synchronized follicular wave to GnRH, exhibiting luteolysis to PGF2α, manifesting estrus and an ovulatory follicle to the treatment of exogenous progesterone can serve as indicators of effective estrous synchronization regimes.

Ultrasonographic observation on ovarian structures can help in choosing effective protocols presynchronization, reduce the variation exhibited during the synchronization process, and observe and evaluate the response to the treatment, thereby facilitating an opportunity for strategic intervention at each phase of the synchronization process to improve the success. Figures 9.13 and 9.14 show grey-scale ultrasonographic images of preovulatory follicle and corpus luteum development and Fig. 9.15a–c shows color-flow Doppler ultrasonographic features of luteal blood flow pattern during different stages of CL development (Farooq and Jeyakumar 2021).

9.13 Use of Estrous Synchronization Protocols in Ovarian Disorders

The risk of high productivity and negative energy balance predispose the dairy herd to ovarian abnormalities and limit their breeding value. Under gynecological diseases, ovarian diseases occupy the major reason for culling from the dairy herds. The common ovarian diseases affecting dairy herd are seasonal ovarian hypofunction, anestrous, cystic ovarian degeneration, and persistent corpus luteum
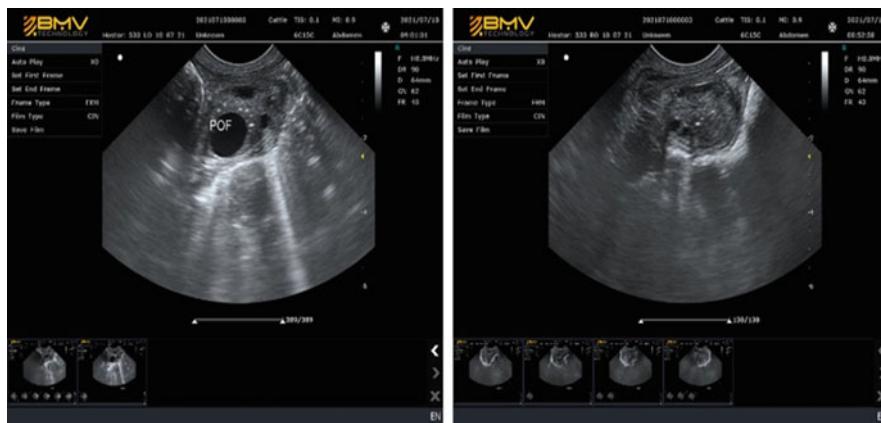
Fig. 9.13 Sonogram of ovaries of Deoni cow (Bos indicus) showing preovulatory follicle on left ovary

(Khamitova et al. 2020). Appropriate hormonal therapy in the initial stage of the disease with balanced feeding can have a beneficial therapeutic effect improving the farm economy.

9.13.1 Anestrous and Acyclicity

The estrus synchronization regimes have been widely employed to treat ovarian diseases with considerable success. With a single injection of GnRH, estrus induction response achieved was 45.5–87.5% spread over a period of 4 to 22 days due to the presence of follicles at different stages of growth (Kumar et al. 2014). The Ovsynch protocol formulated by Pursley et al. (1995) is more promising in the treatment of seasonal anestrus and postpartum anestrus in cattle and buffaloes. The GnRH on day 0 causes recruitment of a new follicular wave by secretion of FSH. In the presence of a dominant follicle, it causes ovulation or luteinization resulting in a CL. The PGF2α on day 7 induces luteolysis of the formed CL followed by ovulation of newly formed dominant follicle by GnRH on day 9. This protocol gives promising results by a reduction in the service period and intercalving period. However, the pregnancy rate with Ovsynch protocol is considerably low, which can be improved by progesterone supplementation with Ovsynch protocol (McDougall 2010). Overall, a 12% increase in the first service conception rate was observed with the addition of progesterone to the Ovsynch protocol. It is because when the first GnRH injection of the Ovsynch protocol fails to induce the dominant follicle to ovulate, the progesterone concentration during the subsequent follicular growth lies below 1 ng/mL resulting in the altered follicular microenvironment and poor conception rates. The exogenous progesterone supplemented during this phase mimics the luteal phase of the normal estrous cycle and leads to a better conception rate (McDougall 2010).
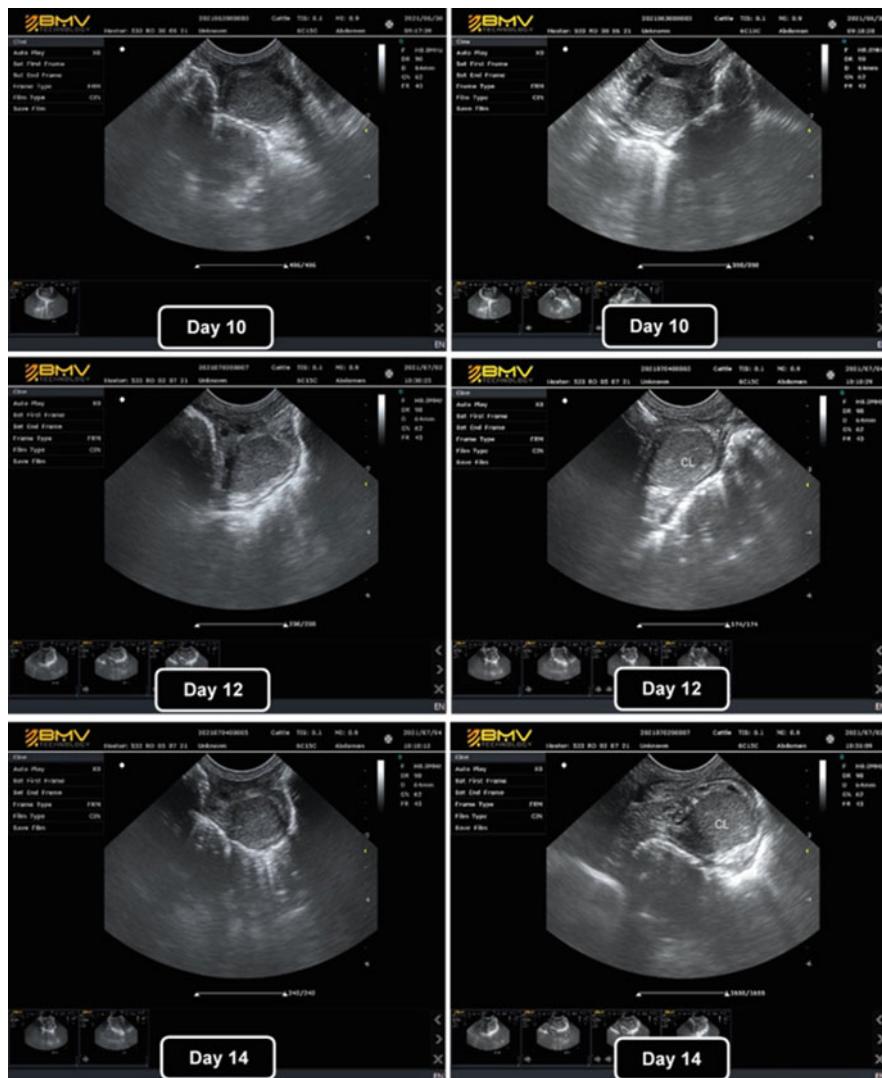
Fig. 9.14 Sonogram of ovary of Deoni cow (*Bos indicus*) showing mature CL (10–14 days)—large size, well-defined borders, granular structure, and hypoechogenic arrangement

The estrus induction efficiency of different estrus induction regimes such as Ovsynch, CIDR, Ovsynch+ CIDR, and Heatsynch evaluated in postpartum anestrous Kankrej cows revealed estrus induction response greater in CIDR followed by Ovsynch, Ovsynch plus CIDR, and Heatsynch. The conception rate at induced heat was highest for CIDR followed by Ovsynch plus CIDR or Ovsynch. The overall conception rate was also highest in CIDR group. However, the mean plasma
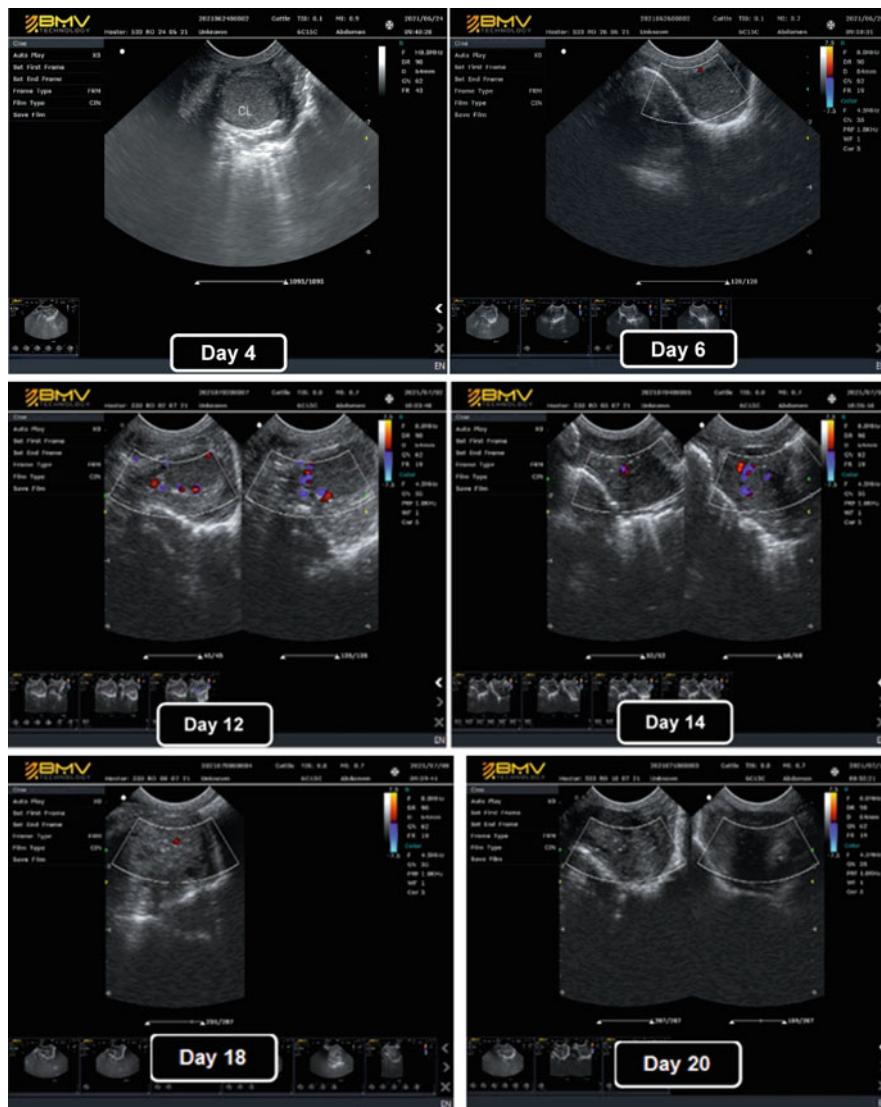
Fig. 9.15 (**a**) Color-Flow Doppler ultrasonography showing luteal blood flow during estrous cycle: early CL—no vascularization. (**b**) Sonogram of ovary showing active/functional CL with high blood vascularity

progesterone concentration was nonsignificantly higher in Ovsynch group compared to CIDR group (Bhoraniya et al. 2012; Naikoo Mehrajuddin et al. 2016). Therefore, among different protocols, use of CIDR serves as the most effective regimen for estrus induction in postpartum anestrous suckled cows with an improvement in the conception rate.

9.13.2 Ovarian Cysts and Persistent Corpus Luteum (PCL)

The failure of ovulatory signal and follicular atresia at the appropriate time during the estrous cycle results in cystic ovarian degeneration. Progesterone intravaginal inserts mimic the luteal support and suppress the LH pulse frequency. The withdrawal of LH support disrupts the endocrine environment required for the maintenance of follicular cysts resulting in the reduction of their size and regression. With the sudden progesterone removal, the normal follicular phase is stimulated resulting in estrus (Jeengar et al. 2018). The progesterone device combined with estradiol or GnRH results in an estrus induction rate of almost 80–100%. Despite optimal estrus induction rates, the progesterone treatment fails to result in optimal conception rates due to aged ovum, altered follicular growth and atresia, poor transport of gametes, resulting in fertilization failure and early embryonic death (Kumar et al. 2014). A weak luteolytic signal or strong luteotrophic signal plays an important role in the pathogenesis of persistent corpus luteum (PCL). Single shot PGF2α can successfully induce luteolysis in cases of PCL and luteal cyst. The administration of PGF2α had a success rate of about 85% and 77% resulting in induction of estrus followed by a first service conception rate of 71% and 57% in PCL and luteal cyst, respectively (Lashari and Tasawar 2012; Borş et al. 2018).

The Ovsynch protocol is a better therapeutic strategy as it can be used in both types of ovarian cysts (follicular and luteal) and similar conception rate to that of timed AI is achievable (Bartolome et al. 2000). The progesterone device combined with GnRH or estradiol results in an estrus induction rate of almost 80–100%. Progesterone intravaginal inserts mimic the luteal support and suppress the LH pulse frequency. The withdrawal of LH support disrupts the endocrine environment required for the maintenance of follicular cysts resulting in the reduction of their size and regression. With the sudden progesterone removal, the normal follicular phase is stimulated resulting in estrus (Jeengar et al. 2018). Despite optimal estrus induction rates, the progesterone treatment fails to result in optimal conception rates due to aged ovum, altered follicular growth and atresia, poor transport of gametes, resulting in fertilization failure and early embryonic death (Kumar et al. 2014). Single shot of PGF2α can successfully induce luteolysis in cases of PCL and luteal cyst. A weak luteolytic signal or strong luteotrophic signal plays an important role in the pathogenesis of persistent corpus luteum (PCL). The administration of PGF2α had a success rate of about 85% and 77% resulting in induction of estrus followed by a first service conception rate of 71% and 57% in PCL and luteal cyst, respectively (Lashari and Tasawa et al., 2012; Borş et al. 2018).

9.14   Conclusion

The advent of newer molecular technologies and application of real-time high-resolution ultrasonography with image processing tools has revolutionized our understanding with respect to the events associated with estrous cycle (follicular and luteal dynamics), which forms the basis for manipulation of estrous cycle in

cattle and buffaloes. The holistic animal husbandry approaches augmented with estrus synchronization programs involving isolated or combined use of prostaglandin, progesterone, GnRH and the recently arrived aromatase inhibitors can greatly improve the reproductive efficiency of cattle and buffaloes. Presently, there is a compelling demand for designing cost-effective estrus synchronization regimes with minimal animal handling for broader farmer's/consumer acceptance that would outspread the use of controlled breeding technology in cattle and buffaloes worldwide.

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Uterine Infection in Bovines: An Update

10

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Abstract

Optimum fertility is a pre-requisite for enhancing productivity as well as lifetime production of dairy bovine. However, increasing evidences indicate that bovine fertility has shown a declining trend over last few decades. Analysis of the existing information indicates that dairy animals calve at a later age, conceive at very later stage of postpartum period, and the conception rate is very low. Among the several reasons for extended service period (in turn calving interval), postpartum uterine infection solely accounts to around 30%. Infectious diseases affecting reproduction have been shown to be associated with losses throughout the reproductive cycle by reducing the rates of ovulation, fertilization, and embryonic and foetal survival. Calving problems, metabolic diseases like milk fever, ketosis, and altered endocrine *milieu* affecting the likelihood of elimination of bacteria could be predisposing factors leading to uterine infections in dairy cows. The conception rate drops down by 20% in cows with uterine infection, the calving to conception interval is extended by 30 days, and 3% more animals are culled for failure to conceive. Therefore, understanding the molecular basis of uterine disease and identification of biomolecules/cues to predict ensuing uterine infection at an early stage, and developing effective preventive and therapeutic

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strategies are the needs of the hour to realize the dream of obtaining a calf per cow per year.

10.1   Introduction

One of the chief reasons for diminished lifetime milk production in an individual dairy cattle is temporary loss of fertility or infertility. The aetiologies of infertility in dairy cattle are many and can be complex. They relate to puberty, sexual maturity, successful insemination, ovulation, fertilization, implantation, development and delivery of normal foetus and foetal membranes, proper uterine involution, resumption of ovarian cyclicity, postpartum oestrus expression, and conceive again at right time. Anything interfering with the routines of this cycle, such as diseases, improper nutrition, poor herd management, endocrine disturbances, or changes in the micro-environment, makes the animal infertile, if only temporary in occurrence. In general, the causes of infertility in dairy animals can be classified into congenital, morphological, functional, infectious, and unknown causes, among which postpartum uterine infections solely account to approximately 30%. The key for exceptional fertility in dairy cattle herds is a good uterine environment because any disorders of endometrium perturb the normal reproductive functions and lead to subfertility or infertility (Sheldon et al. 2009; Jabbour et al. 2009).

Postpartum uterine infection is the usual cause of infertility in cows and has been related with delay in involution of uterus, extended time until first postpartum oestrus, greater number of services per conception, and extended calving to conception interval leading to economic losses. After parturition, uterine bacterial contamination occurs in almost 90 per cent of the animals, which is then eliminated during the process of involution that takes about 4–6 weeks. However, certain percentage of animals (25–30%) are unable to eliminate the infection and suffer from uterine diseases leading to negative consequences on postpartum fertility. The progression of uterine disease depends on the immune status of the cow, the species, and number (challenge or load) of microbes. As the postpartum uterus has tissue debris, which can help bacterial growth, and a disturbed surface epithelium in communication with lochia, the infection gets established easily if the immunity of the cow is compromised.

A normal postpartum cow clears uterine infection by expulsion of uterine content, speedy uterine and cervical involution, and deployment of natural host defences that includes antibodies, mucus, and phagocytic cells. If the animal has altered immune status, then the uterine infection would persist and hence result in varying degrees of metritis. Cows that are having problem in eliminating pathogenic microorganisms acquire uterine infections, viz., puerperal metritis, clinical metritis, or endometritis, depending upon severity of infection and immune status of animals (Sheldon 2004;

Chapwanya et al. 2009). The incidence of uterine infections ranges from 13 to 48% in cattle and buffaloes (LeBlanc et al. 2002a, 2002b; Kasimanickam et al. 2004; Hammon et al. 2006; Huzzey et al. 2007; Azawi 2008). Increasing incidence of postpartum uterine infection compromises the reproductive efficiency of dairy animals. Moreover, recent evidences suggest that uterine infections not only affect the production capacity of the animal, but also affect the follicular development, oestrogen production, oestrus expression, and ultimately the conception, thereby prolonging the calving interval. It has been reported that the cows affected with endometritis had lower conception rates (by 20%), extended calving to conception interval (by 30 days), and about 3% of animals were culled for conception failure (Borsberry and Dobson 1989; LeBlanc et al. 2002a, 2002b; Kasimanickam et al. 2004).

Uterine diseases are generally caused by a range of pathogenic bacteria including *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, *P. melaninogenicus,* and other anaerobic bacteria and viruses. These infections damage the endometrium lining the uterus and ultimately compromise the fertility of affected animals. A compromised systemic and uterine immune function at calving is an important factor for increasing the host susceptibility to postpartum uterine infection. Peripheral immunity including both adaptive and innate immunity plays critical role during transition period and compromised function of this system contributes to the risk of development of uterine diseases. In spite of several studies on the relationship of inflammatory molecules with uterine diseases, defined studies indicating the cause and effect relationship between uterine immune status immediate to parturition and its relation to development of postpartum uterine infections are few. Therefore, a better understanding of the pathogens causing uterine infection, host immunity, impact of uterine infection on production and reproduction, and identifying risk factors associated with development of uterine infection would help in developing effective preventive and therapeutic strategies for downsizing the incidence of uterine infection in dairy animals to ensure increased production and profitability besides supply of safe milk and milk products to the consumers. In this chapter, pathogenesis and impact of uterine infection on production and reproduction in dairy animals are discussed along with pre, peri, and postpartum management strategies to reduce the occurrence of uterine infection.

10.2 Development of Uterine Infection

Bacterial contamination of uterus in cattle is dynamic, wherein contamination and clearance of bacteria and re-contamination occur spontaneously during first 3–4 weeks of postpartum, rather than just at the time of parturition. Various species of aerobic and anaerobic bacteria (both Gram-negative and Gram-positive) have been isolated from the uterus of early postpartum cows. Many of these environmental pathogens are slowly removed during the first 6 weeks after calving. Thus, the existence of bacteria in the uterus of postpartum cows doesn't culminate into uterine inflammatory conditions at all the times. However, the uterine diseases get

established when the pathogen infiltrated to the epithelium or get attached to the mucosa or colonized and liberating bacterial enzymes, toxins, etc. (Sheldon et al. 2009).

The uterine infection develops when the equilibrium between the uterine immune response of cow and the load of the causative microbes and the virulence factors is altered. The presence of bacteria in the uterus leads to histological aberrations of the endometrium and inflammation and postpones the involution of uterus. The severity of inflammation and persistence of infection depends on several factors including type of bacteria present, amount of infection, genetic factors, existence of an appropriate uterine milieu, and innate and acquired immunity of an animal. Besides bacterial load, the virulence factors (VF) are the vital pathogen-related determining factor for development of uterine infection (Gilbert et al. 1998; Sheldon et al. 2009). Even though the various range of bacteria were isolated from uterus during the first 3 weeks after calving, the existence of *Escherichia coli, Arcanobacterium pyogenes, Prevotella melaninogenicus,* and *Fusobacterium necrophorum* is usually related with severe inflammation of endometrium and extreme clinical disease of postpartum cows (Griffin et al. 1974; Bonnett et al. 1991; Bicalho et al. 2010).

It has been reported that six VFs of *Escherichia coli* were related with uterine diseases: hlyA, kpsMII, fimH, cdt, astA, and ibeA (Bicalho et al. 2010). The fimH was the predominant reported VF gene in cows having metritis. Besides *Escherichia coli,* numerous studies highlighted *Arcanobacterium pyogenes* as the aetiology of infertility and endometrial damage in postpartum cows (Dohmen et al. 2000). Further, it was stated that successive contamination of the uterus after calving by the common bacteria could regulate the occurrence and severity of uterine infection. For example, infection on first week postpartum by *E. coli* rises the vulnerability of the endometrium to succeeding *A. pyogenes* infection (Olson et al. 1984; Williams et al. 2007), and *A. pyogenes* works synergistically with *Provetella sp.* and *F. necrophorum* to enhance the severity of the disease (Bonnett et al. 1991). *E. coli* produces bacterial cell-wall constituents lipopolysaccharide (Williams et al. 2008); *A. pyogenes* releases the growth factor for *F. necrophorum* and pyolysin - a cholesterol-dependent cytotoxin (Sheldon and Dobson 2004; Bicalho et al. 2012); *Provetella sp* releases a phagocytosis inhibiting element and *F. necrophorum* releases leukotoxin (Sheldon and Dobson 2004). Therefore, in addition to host aspects, the VFs of the common endometrial pathogens or/and load of bacteria regulate the postpartum uterine infections and culminate in subclinical or clinical endometritis.

10.3   Classification of Uterine Disease

The outcomes of uterine infections of early postpartum cows include puerperal metritis, clinical endometritis, pyometra, and subclinical endometritis. Clinical definitions for common postpartum uterine infection, proposed by Sheldon et al. (2006), are given below.

- Puerperal metritis, also denoted as septic metritis, toxic metritis, or acute metritis, is an infection of the uterus that causes acute systemic illness. The affected animals show an enlarged uterus and a reddish-brown watery fetid uterine discharge, systemic signs of illness (dullness, drop in milk yield, or signs of toxemia), and fever (>39.5 °C), within 21 days after calving. In extreme cases, decreased milk yield, obvious dehydration, inappetence or anorexia, dullness, and increased heart rate may also be present.
- Animals affected with clinical metritis do not display any systemic illness; however, the uterus is enlarged and purulent uterine discharge is noticeable in the vagina, within 3 weeks postpartum.
- Clinical endometritis is not associated with systemic signs, but is characterized by the existence of mucopurulent (approximately 50% pus, 50% mucus) or purulent (>50% pus) discharge in the vagina by 21 days or more after calving.
- Subclinical endometritis is defined as inflammation of the uterine endometrium with no clinical signs associated with it. The condition is usually diagnosed by uterine cytology (by measuring the proportion of neutrophils in samples obtained using a cytobrush or uterine lumen flushing) in the absence of detectable purulent material in the vagina.
- Pyometra is distinguished by the abnormal distension of the uterus, because of accumulation of mucopurulent or purulent substances, in the presence of a corpus luteum.

Clinical endometritis and metritis affect around 20% of lactating dairy cows; the prevalence ranges from 5.0 to >30% and 8 to >40%, respectively (LeBlanc et al. 2002a, 2002b; Hammon et al. 2006; Huzzey et al. 2007; Galvao et al. 2009). Subclinical form of uterine infection (Subclinical endometritis) is the most common with the prevalence rate of around 30% among lactating dairy cows; however, the incidence could range from 11 to >70% in some herds (Gilbert et al. 2005; Kasimanickam et al. 2004). Around 18–40% of cattle and buffaloes were culled due to infertility in India (Sharma et al. 1993). Earlier studies from India reported that the incidence of uterine infection, metritis, and endometritis was 24.7% (Rao and Sreemannarayana 1983), 25% (Sar et al. 1996), and 30% (Rao 1982), respectively. A recent report indicated that the incidence of metritis was 10.32% and 22.56%, in Zebu cattle and crossbred, respectively (Kumari et al. 2016). The reported incidence of endometritis in buffalos in Iran was 33.2% (Moghami et al. 1996). In Egyptian buffaloes, Ghanem et al. (2002) recorded that the incidence of endometritis was 22.4%. Overall, a higher prevalence of uterine infection has been reported in buffaloes than in cows.

10.4 Impact of Uterine Infection on Production and Reproduction

The economic effect and the incidence of subclinical and clinical endometritis in postpartum cow was evaluated by several studies. Uterine infection is associated with lower milk yields, and if associated with retained foetal membranes, then the magnitude of milk loss is further high. It has been shown that the cows affected with severe and mild metritis produced 8.3 and 5.7 kg/day lesser milk compared to the normal cows during the first 3 weeks of calving, respectively. Metritis has a negative impact on the success rates of the successive inseminations and hence indirectly affects the lifetime production of the cow. The puerperal metritis increased the days open, days to first insemination, number of inseminations/conception significantly by 46.16 days, 18.24 days, and 0.54, respectively (Garcia et al. 2002a, 2002b). Metritis-associated poor reproductive consequences lead to culling of 25% and 42% of low milk yielder and high yielder cows, respectively. The risk of culling in metritis-affected cows ranges from 1.2 to 1.7 times. The milk yield in buffaloes in single lactation was reduced by 173 kg for dystocia, 239 kg for retained foetal membrane, 98 kg for metritis, and 181 kg for stillbirth (Kumari et al. 2016).

Uterine infection in dairy cows may affect the endometrial regeneration and interrupt the recommencement of ovarian cyclicity leading to delayed first insemination, extended calving interval, reduced calving rates, and increased number of inseminations per conception. Additionally, lower submission rates were reported in cows with a pus-filled uterine discharge. Infection with bacteria leads to inflammation and damaged endometrium, diminishing the chances of conception. In response to the bacterial pathogen-associated molecules, the endometrial cells of cows secrete chemokines and cytokines to elicit the immune reaction. The neutrophils and macrophages are invited by chemokines for bacteria removal. Nevertheless, at the time of parturition in cattle, the functions of neutrophil are often compromised. The existence of polymorphonuclear leukocytes even when the bacteria is absent in the endometrium is considered to be the principal representation of subclinical endometritis. In the animals with uterine infection, the quantity of follicle stimulating hormone (FSH) secreted from the pituitary remains unaffected, therefore the ovarian follicular waves arise in the first week postpartum. But, the release of Gonadotropin-releasing hormone from the hypothalamus and the release of Luteinizing hormone (LH) from the pituitary are repressed in animals with uterine infection leading to alterations in ovulation of the dominant follicle.

Recent investigations reveal that lipopolysaccharide (LPS) derived from infection of the uterus with Gram-negative bacteria was shown to suppress steroid production in the granulosa cells of follicles. In the animals with uterine infection, the follicular fluid contains increased LPS concentrations that in turn reduces the expression of aromatase, an enzyme required for conversion of testosterone into estrogen. Recently, it has been reported that even low concentrations of LPS in follicular fluid may inhibit follicular activity by suppressing transcriptions of steroidogenic enzymes such as CYP17 and P450aromatase. Moreover, LPS in follicular fluid may related with follicular atresia (Magataa et al. 2014). Therefore, these animals have
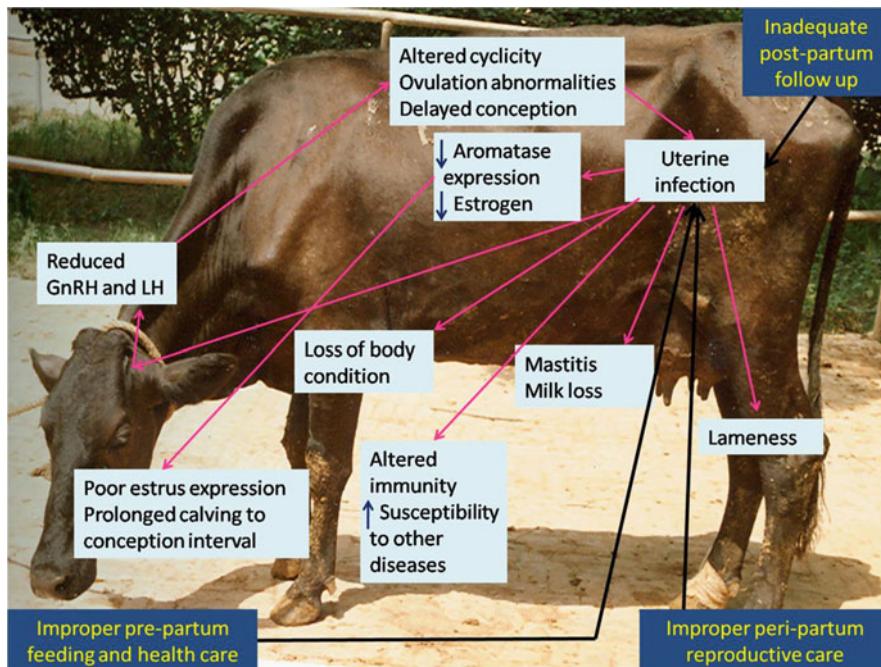
Fig. 10.1 Diagram depicting the effects of uterine infection on reproductive events in cattle

lower peripheral plasma estradiol concentrations and so have less chance to ovulate due to poor positive feedback of oestrogen to the LH. Despite these, if ovulation occurs in endometritis-affected cows, the corpus luteum formed has reduced ability for progesterone production. Nevertheless, the progesterone concentrations in peripheral plasma are lesser in cows affected with uterine infections as compared to healthy fertile animals. The inflammatory cytokines produced in response to the bacterial pathogen-associated molecules may perturb luteal cell steroidogenesis. Luteolysis is also disrupted in the animals with uterine infection since the endometrial epithelial cells of infected animals secrete principally PGE (anti-inflammatory) rather than PGF (luteolytic), and luteal phases are often extended. The effect of uterine infection on reproductive physiology is depicted in Fig. 10.1.

10.5 Losses Associated with Uterine Infection

The economic loss related with uterine infection is reliant on reduced milk yield, the cost of treatment, and subfertility. In an estimate from the USA, it is reported that the single case of metritis accounts for the economic loss of between $304 and $354 by impacting the performance and production. The magnitude of the estimated loss due to uterine infection may surprise some, but the reality is that uterine infection is an expensive problem in dairy bovine. If one applies these estimates for a 1000-cow

herd with a 20% incidence, the loss would be around $66,000 per year (Overton and Fetrow 2010). In the UK, a direct loss of £62 for reduced milk yield and that for treatment of a cow with uterine disease of £62 and the indirect loss of £69 for the increased culling rate, longer calving interval, lower oestrus expression, and extra inseminations per cow have been estimated. The estimated annual direct costs of uterine disease were £725 for 100 cows for 21 dairy herds (Esslemont and Kossaibati 2002).

10.6 Risk Factors for postpartum Uterine Infection

The risk factors for uterine disease include altered gestation length, twin pregnancies, stillbirth, retained foetal membranes, and assisted parturition. Previously, it was stated that the immediate causes (during immediate peri-partum period) for postpartum uterine infection were retention of foetal membranes and intrauterine manipulation, etc. But recent evidences suggest that the pre-partum animal's well-being has vital part in influencing the animal to the progression of postpartum uterine diseases. The risk factors for uterine infection in cattle and buffaloes are given in Fig. 10.2.
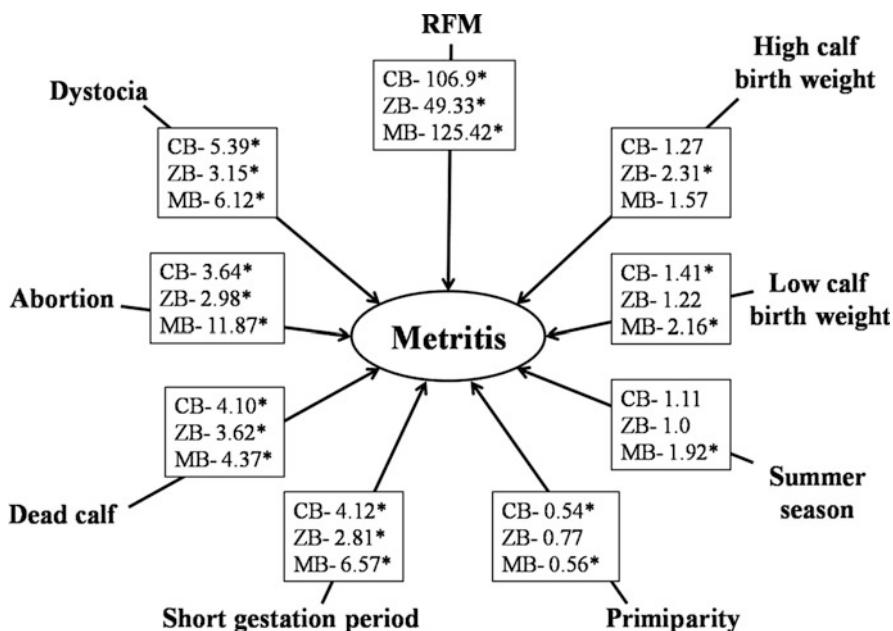

Fig. 10.2 Odds ratio estimate of risk factors for metritis in cattle (crossbred and zebu) and Murrah buffaloes. *CB* crossbred cattle, *ZB* zebu cattle, *MB* Murrah buffaloes; * significant $P < 0.05$ (From Kumari et al. 2016)

10.6.1 Nutrition

Energy status during the puerperium is an important risk factor for the postpartum uterine disease. Following calving, metabolic imbalance, in particular negative energy balance (NEBAL), interferes with the uterine involution because of its immunosuppressive effect. Clearly, the energy metabolites, non-esterified fatty acids (NEFA), and beta-hydroxybutyric acid (BHBA) accompanied with decreased body condition score (BCS) are the indicators for NEBAL. Several authors worked in this line; in specific, Hammon et al. (2006) reported that the cows under NEBAL during the peri-partum period are predisposed to metritis or endometritis because of the fact that the function of neutrophil is hampered due to low glycogen reserves (Galvão et al. 2010), leading to an immune compromised state. Energy metabolites, NEFA and BHBA, could be considered as important risk factors for the postpartum uterine disease because several studies revealed that an increased concentration of these metabolite levels during pre-partum resulted in postpartum metritis and endo-metritis (Hammon et al. 2006; Galvão et al. 2010; Giuliodori et al. 2013). The low energy status during the peri-partum period and early lactation could be considered as one of risk factors for subclinical endometritis (Burke et al. 2010). Further, some studies reported the cows with high concentrations of energy metabolites (NEFA or BHBA) during transition period may cause alterations in gene expression of pro-inflammatory cytokines (IL1 and IL8) in the uterus (Wathes et al. 2009a, 2009b).

The energy requirements are increased during the final month of gestation, but on the other hand, the feed intake is usually decreased. During immediate postpartum period, the cows are prone for several complications including the metritis, if the animal's energy requirements are not met with energy density diet. As pregnancy advances, the protein demand for the foetal growth also increases. When the protein intake is less than the required amount during final 3–6 weeks pre-partum, the incidence of retained foetal membranes, ketosis, and infertility increases. There are clear evidences that the changes in blood metabolites and metabolic hormones during pre-partum period could act as signals to the reproductive system to reduce fertility and make the animal prone for developing metritis. Recommended levels of vitamins A and E, selenium, and calcium in the diet of cows are vital. The reduced calcium in blood may contribute to retained placenta, as it is vital for smooth muscle contraction of uterus, leading to delayed uterine involution and uterine infection. Many other minerals are also responsible for uterine health maintenance; therefore, unbalanced ration for the dry cow would predispose the cow to metabolic and associated complications making the cow at risk of developing metritis.

10.6.2 Environment

During the transition period (3 weeks before and 3 weeks after calving), the impact of stress is augmented, therefore care should be taken for cooling in hot climates, stocking rates, and cow comfort. *Escherichia coli, Arcanobacterium pyogenes,*

Prevotella, and Fusobacterium necrophorum are the commonly linked species of bacteria with clinical endometritis. These organisms are acquired presumably from the faecal contamination of the animal coat, environment, and bedding. Nevertheless, the involvement of the faecal contamination and environment in increasing the risk for clinical endometritis is usually ignored. During calving and few days after, the uterus is prone for the invasion of many infectious organisms in the environment including bacteria, moulds, and viruses due to dilated cervix. Contamination in the stanchion areas or maternity pens offers a perfect milieu for reproductive tract diseases. Additionally, the reduced functioning of immune system around calving makes the cow susceptible to many infections.

10.6.3 Calving Assistance

Assistance should be given to the cows during calving only when it is absolutely needed. Needless and inappropriate assistances make the animal prone for uterine diseases. Assistance too early during the calving may lead to serious complications. In order to avoid complications during calving, the size of heifer should be considered before insemination. Dystocia can be avoided by inseminating heifers with the semen from calving-ease sire.

10.6.4 Peri-Partum Complications

The main metabolic complications during peri-partum are milk fever, displaced abomasum, and ketosis. The peri-partum conditions predisposing for uterine disease include dystocia and retention of foetal membranes (RFM). RFM has been the most important risk factor for development of uterine infection and its association with metritis is consistently reported. Abortion is the chief predisposing factor for RFM and the other factors predisposing the animal to RFM include stillbirth, milk fever, dystocia, pluriparity, twin births, shorter gestation length, and winter season of cows. RFM and milk fever are the chief risk influencers for metritis.

10.7   Identification of Cows at the Risk of Developing postpartum Uterine Infection

With the advances in scientific techniques, it has become possible to identify pre-partum bio-markers that could fairly predict the risk of developing postpartum metritis. These markers include pre-partum feeding, drinking and social behaviour of animals, and metabolites in blood like β-hydroxy butyric acid (BHBA) and non-esterified fatty acid (NEFA), etc.

Behavioural markers: The feeding time and bouts during pre-partum period can be good a behavioural marker for forecasting the metritis development during postpartum as the cows that developed postpartum metritis had significantly reduced

prepartum feeding time and bouts (Patbandha et al. 2013). Reduced feeding time and bout indirectly indicates the lower intake of dry matter by metritic cows along with the risk of changed circulating ketone bodies and free fatty acids during transition period. This impairs the function of neutrophils and hence make the cows prone for postpartum uterine infections. In addition, socially, the cows affected with mastitis are subordinate during prepartum, so they are displaced more by others compared to normal cows indicating that the pre-partum health of the animal is not very sound.

Biochemical markers: Negative energy balance in dairy cows during the transition period has been regarded as a key risk factor for increased incidence of metabolic disorders. Decreased energy intake during pre-partum period increases the mobilization of adipose tissue as parturition approaches resulting in increased NEFA concentrations. The rise in plasma NEFA around the time of parturition is partly due to hormonal changes and stress associated with parturition and reduced DMI during transition period. A rapid increase in plasma NEFA occurs in the last 3 days of gestation. Negative energy balance during transition period has been shown to be related with development of postpartum metritis. During peri-partum period, the NEFA and BHBA concentrations were reportedly higher significantly in metritic cows compared to normal cows (Patbandha et al. 2012). Similarly, the NEFA:total cholesterol ratio in plasma has been shown to be significantly more in metritic cows throughout the peri-partum. These biochemical markers can be used for identifying dairy cows that are prone for development of postpartum uterine infection. The biochemical markers based "on-spot" diagnostic kits are available and are being used for early detection of subclinical ketosis and other metabolic disorders, which can help identifying the cows at risk of developing metritis at an early stage so that effective management strategies can be applied to prevent postpartum uterine infection and relate to consequences.

10.8 Diagnosis

Cows that develop metritis within 21 days postpartum, if not diagnosed at time and remain untreated later, develop clinical endometritis and fertility is compromised. Therefore, strategic approach should be taken to monitor and diagnose cows that are developing clinical or puerperal metritis. Monitoring the rectal temperature of dairy cows daily up to 10 days postpartum and examination for abnormal uterine discharge on first, second, and third week postpartum are helpful to diagnose puerperal infection at earliest and make their treatment possible with increased cure rate and improve the subsequent reproductive performance of dairy cows.

The evaluation of vaginal content for the presence of pus is highly valuable and frequently used for diagnosing uterine diseases. Commonly, the diagnosis of clinical endometritis is by the uterine discharge assessment detected in the vagina with the help of either gloved hand, metricheck device, or speculum (Williams et al. 2005; McDougall et al. 2007; LeBlanc et al. 2002a, 2002b). The diagnosis of clinical endometritis relies on appropriate visual characterization of the cervico-vaginal or uterine discharge. The character of the discharge can be scored based on appearance

Table 10.1 Diagnostic method(s) used for evaluation of bovine endometrial inflammation

S. No.	Diagnostic method	Reference
1.	Per rectal examination	LeBlanc et al. 2002a, 2002b
2.	Vaginoscopy	Gilbert and Schwark (1992)
3.	Uterine biopsy	Chapwanya et al. (2010)
4.	Low volume uterine lavage	Galvao et al. (2009), Santos et al. (2009)
5.	Endometrial cytology	Couto et al. (2013), Pascottini et al. (2016)
6.	Ultrasonography	Kasimanickam et al. (2004)
7.	Bacteriology	Sens and Heuwieser (2013)
8.	Exploration of blood metabolic inflammatory mediators	Heidarpour et al. (2012), Akbar et al. (2014)
9.	Assessment of enzymes	Cheong et al. (2012), Couto et al. (2013)

as well as odour. Odour has been scored from 0 to 3, i.e. Score 0 for no odour and Score 3 for a fetid odour (Williams et al. 2005). Character of the mucus is scored using 0–3 scale (Score '0' = normal uterine discharge, Score '1' = purulent flakes in the uterine discharge, Score '2' = <50% purulent exudate in the uterine discharge, Score '3' = >50% purulent exudate mixed with uterine discharge).

Additionally, evaluating the uterine and cervical involution using ultrasonography will give an indication about the uterine health status. Healthy cows have 3–4 cm of uterine horn diameter by 25–30 days after calving and < 5 cm diameter cervix by 40 days after calving, but uterine and cervical involution is not completed until approximately 50 days post-calving (Sheldon 2004). Nevertheless, several factors including age, nutrition, and miscellaneous factors affect uterine involution. Therefore, the delay in uterine involution is not a precise indicator of uterine disease. The commonly used diagnostic methods for detecting endometrial inflammation are indicated in Table 10.1. A collective review of the literature has shown that no cow-side test has until now been established for subclinical endometritis (Pascottini et al. 2016). However, few tests could be used either alone or in combinations to have an indication about the subclinical uterine infection, which are indicated below.

10.8.1 Whiteside Test

In field conditions, especially in smallholder production system, whiteside test could be used to ascertain the grades of non-specific genital tract infections. Briefly, 1 ml of estrual cervico-vaginal discharge is heated with the equivalent volume of 5 to 10% sodium hydroxide till boiling point followed by cooling the sample. The colour intensity depends on the severity of endometritis and concentration of leucocytes which is categorized as normal (no colour), mild (light yellow), moderate (yellow colour), and severe infection (dark yellow colour) (Bhat et al. 2014).

10.8.2 Endometrial Biopsy

Undoubtedly, endometrial biopsy is considered to be the gold standard method for detection of endometritis. Endometrial biopsies are graded based on the rate of recurrence, inflammation status, periglandular fibrosis, and glandular nesting. In acute inflammation, neutrophils are the prime indicators, whereas lymphocytes are the prominent feature in chronic inflammation (Rosales 2018). Previous studies reported that this procedure itself is harmful and induces uterine pathology and affects the future fertility of the animal (Zaayer and van der Horst 1986). However, a recent study reported that this procedure is safe and reliable for the assessment of postpartum uterine health of cows, if it is carried out properly (Chapwanya et al. 2010).

10.8.3 Endometrial Cytology

Although endometrial biopsy appears to be the better method to diagnose subclinical endometritis, biopsy procedure itself may cause damage and inflammation to the uterine lining. Alternately, uterine cytology (Fig. 10.3) can also be used for the diagnosis of subclinical endometritis. Endometrial cytology technique involves the measuring of the percentage of neutrophils in the collected uterine samples from low volume uterine lavage technique (Gilbert et al. 2005) or from cytobrush (Kasimanickam et al. 2004). Subclinical endometritis is characterized by the presence of more than 18 per cent neutrophils (PMN) in uterine cytology of samples obtained during 21–33 days postpartum or more than 10% PMN during 34–47 days postpartum or > 5% PMN between 40–60 days postpartum (Sheldon et al. 2006). A hypothetical model displaying on how the PMN population is varying in normal and SCE cow during early (day 7) and late (day 21-day 60) postpartum is shown in Fig. 10.4. Currently, it is doubtful that endometrial cytology can be used routinely in veterinary practice due to wide variation in the threshold values of neutrophils (5–18%) (Wagener et al. 2017). In addition, sample preparation, sampling
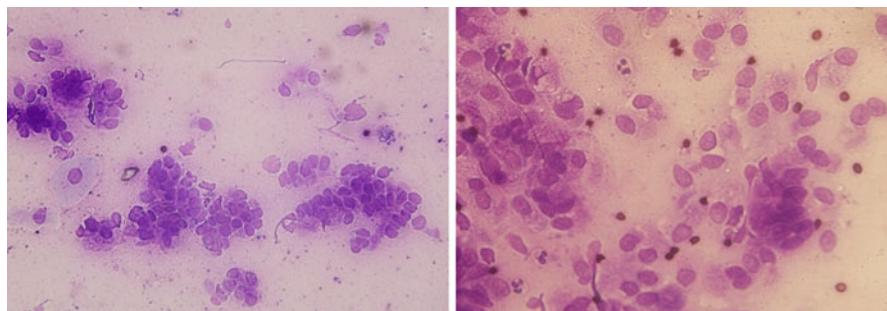
Fig. 10.3 Uterine cytology of normal (left) and subclinical uterine infection (right) affected buffaloes
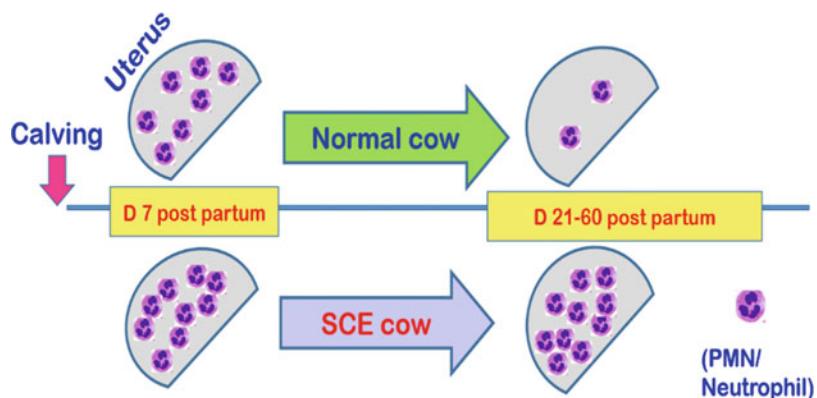
Fig. 10.4 Hypothetical model showing PMN proportion in the uterus of normal and SCE cow following calving

procedure, and evaluation are time-consuming; therefore, it is considered to be the reference method but not an accurate diagnostic test for SCE (Walsh et al. 2011).

10.8.4 Bacteriological Study

Bacteriological studies conducted for determining puerperal metritis and clinical endometritis conditions found that majority of the bacteria were related to non-specific mixed infections that invade the uterus at parturition or shortly after parturition (Sheldon et al., 2002). Recently, Madoz et al. (2014) have conducted bacteriological study for diagnosis of subclinical endometritis in grazing dairy cows and recommended that bacteriology may not be reliable tool as bacteria were not isolated from the cows affected with subclinical endometritis.

10.8.5 Uterine Lavage Sample Optical Density Test

Uterine lavage sample optical density test (ULSOD) is used for diagnosis of clinical endometritis. Briefly, sterile saline solution (20 mL) is infused inside the uterus and mixed gently, and the 5–15 mL of fluid is aspirated and processed for optical density measurement at 620 nm in a 96- well plate microplate reader. This test measures the concentration of proteins or cells in the uterine lavage sample and the recommended threshold for $ULSOD_{620}$ for clinical endometritic animal is >0.058 with specificity of 78%. $ULSOD_{620}$ measurement could be used as a substitute to endometrial cytology diagnostic technique as the former procedure is less laborious than acquiring endometrial cytology details (Machado et al. 2012).

10.8.6 Leukocyte Esterase Test

Leukocyte esterase test (LES) is another recently developed method for detection of subclinical endometritis in uterine flushing fluids of dairy cattle. In this test, the hypothesis is that measurement of the activity of leukocyte esterase, an enzyme produced by neutrophils, reflects uterine inflammation. Although the results of LES-based tests correlated positively with the results of endometrial cytology; to fully recommend the use of LES test as a cow-side diagnostic tool, this method requires additional fine-tuning in sample preparation and processing (Couto et al. 2013).

10.8.7 Intrauterine Oxygen Reductase Potential

Intrauterine oxygen reductase potential is used as an indicator for uterine infection. Either *Trueperella pyogenes* alone or along with anaerobic bacteria such as *Fusobacterium necrophorum* and Bacteroides species is frequently associated with uterine infection and thus leads to a drop in the intrauterine oxygen reductase potential creating an anaerobic environment in the uterus favouring the growth of anaerobic bacteria and aggravating the uterine infection as a whole (Dohmen et al. 1995). This reduced oxygen reductase potential in uterus may be linked with metabolism of microorganisms or excessive oxygen utilization by neutrophils.

10.8.8 Myeloperoxidase Activity and Cytochrome c Reduction Assay

Recent studies demonstrated that cows suffering with uterine health disorders have decreased neutrophil function. The phagocytic capability of neutrophils can be assessed by the cytochrome c reduction assay and myeloperoxidase activity. Myeloperoxidase is released when the phagosome attaches with the neutrophil's primary granules. However, cytochrome c reduction assay evaluates the quantity of superoxide anion formed by the neutrophils in the course of oxidative metabolic burst linked with phagocytosis (Hammon et al. 2006).

10.8.9 Ultrasonography

Nowadays, ultrasonography is an essential tool in regular clinical examination of cattle's reproductive tract. With respect to subclinical endometritis, it is an easy and fast technique available based on the presence of fluid accumulation in uterine lumen. Transrectal ultrasonography could be utilized for diagnosing subclinical endometritis based on the presence of fluid in uterine lumen. However, this method is biased at times due to location of the probe on the uterine horn and endometrial thickness and fluid accumulation is also noticed in non-affected SCE cow at the time of oestrus (Kasimanickam et al. 2004).

10.9 Search for Molecular Markers for Subclinical Uterine Infection

With the aim to develop highly sensitive and specific tests for cow-side detection of subclinical uterine infection, several researchers studied the molecular changes associated with the condition at peripheral and uterine levels. For the benefit of the readers, this information is crystallized here. Gabler et al. (2010) studied the expression of pro-inflammatory genes in the epithelial cells of endometrium (cytobrush-collected samples) in cows positive for uterine disease. They reported an increased mRNA expression of pro-inflammatory mediators such as CXCL5, ILIB, IL8, TNF, and PTGS2 and an acute phase protein, Haptoglobin (HP), from the endometrial samples of clinical and subclinical endometritis cows in a time-dependent manner. This study also demonstrated that mRNA levels of pro-inflammatory mediators and acute phase proteins were correlated with the percentage of neutrophils, where they found peak mRNA levels of inflammatory mediators and high proportion of neutrophils on day 17 postpartum. This research suggests not only the neutrophil evaluation, but also characterization of mRNA expression levels of pro-inflammatory mediators and acute phase proteins could help judging the infection or inflammation status of the bovine uterus following calving. Fischer et al. (2010) reported an upregulated mRNA expression of CXCL5, IL1B, IL8, and TNF during subclinical endometritis and around the time of ovulation and suggested the importance of sample collection during different stages of oestrous cycle. Galvao (2011) studied the mRNA expression analysis of IL-6, IL-1B, TNF, and IL-8 in Holstein cows affected with endometritis and reported IL-1B and TNF pro-inflammatory cytokines levels are reduced during the first week postpartum; however, the levels of IL-6, IL-1B, and IL-8 have shown an amplified pattern during the 5–7 weeks of calving. Duvel et al. (2014) reported higher mRNA levels of chemokine (CXCL8 and CXCL1) and pro-inflammatory cytokine (IL1B) in blood monocytes of subclinical endometritic cows. Peter et al. (2015) reported that transcript levels of pro-inflammatory cytokines (IL1A, IL1B, IL6, TNF, MMP1, and IL8), chemokines (CXCL1, CXCR2, CXCL3, and CXCL5), prostaglandins (PTGS1, PTGS2, PTGIS, PTGES3, and PTGDS), and phospholipid mediator (PTAFR) showed increased levels during late postpartum (45–51 days) than the early postpartum (24–30 days) in subclinical/clinical endometritic cows.

Johnson et al. (2015) also reported a higher mRNA expression of pro-inflammatory mediators (IL1A, IL6, IL17A, TNF, PGHS2, and PGES) in primiparous cows affected by clinical and subclinical endometritis. Recently, Salilew-Wondim et al. (2016) carried out global transcriptome and miRNome analysis in clinical and subclinical endometritic cows during 42–60 days postpartum and presented noticeable alterations in molecular pathways which includes immune system, signalling pathways (G-protein and apoptotic), cell adhesion, and chemotaxis. Baithalu et al. (2017) conducted a study on zebu cows affected with subclinical endometritis, where the changes in blood serum concentrations of oxidants (TAC, MDA, and NO) lead to alteration of genes encoding for antioxidant enzymes (CAT, GPx4, and SOD2) in the uterus, which ultimately compromise the uterine defence

mechanism culminating in postpartum uterine disease. A number of studies described how prostaglandins interact with their synthesizing enzymes during subclinical endometritis (Gabler et al. 2010; Baranski et al. 2013; Peter et al. 2015). For instance, Baranski et al. (2013) reported the pattern of bovine endometrial epithelial cells' secretion of inflammatory mediators (prostaglandins and leukotrines) during clinical and subclinical endometritis. Results of the study showed that the concentration of PGF2α, PGE2, LTB4, and LTC4 were higher in clinical endometritis cows, whereas in subclinical endometric cows, PGF2α concentration was decreased at 18% PMN threshold level. Moreover, PGE2 concentration in subclinical endometritic cows showed an increased level at 18% PMN threshold level, but decreased at 5% PMN threshold level.

Besides cytokines, acute phase proteins, prostaglandins, and antimicrobial peptides (AMPs) form the innate immune defence system in most of the species during physiological and pathological conditions (Zasloff 2002). In addition, AMPs are also expressed in the uterine tissue of healthy and diseased cows in their earlier postpartum (Chapwanya et al. 2009) and form one of the important components of endometrial innate immune defence mechanism (Sheldon et al., 2014). Transcripts of AMPs (LAP, TAP, S100A8, S100A9, and S100A12) showed an increased expression in the bovine uterus treated with E. coli in vitro (Swangchan-Uthai et al. 2012; Chapwanya et al. 2013). Recently, Ibrahim et al. (2016) reported a significant upregulation of AMPs (DEFB4A, DEFB1, DEFB5B, TAP, LAP, S100A12, S100A11, S100A9, S100A8) in clinical and subclinical endometritic cows during 45–51 days postpartum. The above-mentioned AMPs showed an upregulation of their transcripts during the time of ovulation also, which is considered to be an inflammatory event. Few authors reported that the mRNA expression of AMPs at the time of inflammation is dependent on the secretion of inflammatory mediators such as cytokines (Mork et al. 2003; Wathes et al. 2009a, 2009b). A chain of transcript evaluation on pro-inflammatory mediators, acute phase proteins, prostaglandins, and antimicrobial peptides (AMPs) is therefore required in combination to assess the inflammatory condition of the uterus, so they can be employed as diagnostic indicator(s) for uterine diseases postpartum.

Choe et al. (2010) carried out research to discover altered proteins by means of MALDI-TOF-MS mass spectrometry in the uterine washing of clinical endometritic cows. Results from the study showed an upregulation of some of the proteins such as desmin, α-actin-2, heat-shock protein (HSP) 27, HSP 70, collectin-43 precursor, and MHC class I heavy chain (MHC-Ih), whereas transferrin, haemoglobin β subunit, and interleukin-2 precursor were downregulated. Proteomic analysis confirmed the upregulation of Desmin, α-actin-2, heat-shock protein (HSP) 27, and HSP 70 in clinical endometritic cows, which are mainly involved in maintaining the cell stability and integrity. Brodzki et al. (2015) conducted a study to see the levels of pro-inflammatory cytokines (IL6 and TNFα), Interleukins (IL10), and acute phase proteins (SAA and Hp) in the uterine washings and blood of subclinical endometritic cows during late postpartum. Results showed an increased level of TNFα, IL6, IL10, SAA, and Hp in blood; however, in uterine washings, the levels of IL6, IL10, and Hp were significantly increased. These studies highlight the possibilities of identifying

potential molecular markers associated with subclinical uterine infection, which can be used for development of cow-side test for accurate diagnosis of the condition.

10.10 Therapy

An ideal treatment of puerperal infection should be directed towards saving life of affected cows in case of toxic metritis, elimination of bacterial contamination from the environment of uterus and the sub-endometrial layers without hindering uterine defence, and improving subsequent fertility. Parenteral antibiotic administration is the best line of treatment for uterine infection. Several broad-spectrum antibiotics have been used for systemic administration including penicillin, oxytetracycline, and ceftiofur; however, ceftiofur is the antibiotic of choice for the treatment of metritis in dairy cows (Galvao 2011). Ceftiofur is a broad-spectrum third-generation cephalosporin. Long-acting ceftiofur formulations have shown better results because the minimum inhibitory concentration is maintained for 5 days or above against pathogenic bacteria including *A. pyogenes, F. necrophorum,* and *E. coli* in endometrial tissue and blood. The suggested dose of Ceftiofur for metritis treatment in postpartum cows is 2.2 mg/kg body weight. Additional treatment is required in severe cases of puerperal metritis in order to stabilize the animal condition including rehydration therapy either through oral or systemic route and administration of nonsteroidal anti-inflammatory drugs. Even though oxytetracycline is extensively used, that is not an ideal choice because several studies reported the bacterial resistance to oxytetracycline; therefore, high dose is essential for inhibiting the growth of bacteria (Sheldon et al. 2009).

Generally, the therapy for clinical endometritis is on the basis of two approaches; an intrauterine infusion of antibiotics and systemic administration of prostaglandin F2α. The following criteria are vital for selecting antibiotics for intrauterine administration:

- The antibiotic should maintain its action in the uterine environment against the main uterine microorganisms associated with uterine disease. As uterine environment is anaerobic, antibiotics like aminoglycosides are not recommended for the postpartum uterus because it is effective in anaerobic milieu.
- The lochia in uterus contains debris, organic fluids, and various Gram-positive and negative aerobic/anaerobic bacteria (Sheldon 2004). Therefore, a broad-spectrum antibiotic that can elicit its action in the existence of organic debris is advisable for intrauterine administration.
- The antibiotic should maintain MIC against pathogenic bacteria at the place of infection.
- The preparation shouldn't hinder the regular uterine defence mechanism and should not cause endometrial irritation. Most antiseptics and several antibiotics administered through intrauterine route suppress the phagocytic activity of neutrophils even after several days after intrauterine administration (Bretzlaff 1987).

For treatment of clinical endometritis, intrauterine administration of cephapirin benzathine is recommended (LeBlanc et al. 2002a, 2002b). However, a combined treatment of PGF2α and systemic antibiotic shows better effect when corpus luteum is present on surface of ovary as compared to intrauterine administration of antibiotic alone. For subclinical endometritis, intrauterine administration of cephapirin benzathine and/or PGF2α is the treatment of choice as treatment with these drugs enhances reproduction of cows affected with subclinical endometritis (Kasimanickam et al. 2005; Galvao et al. 2009). The advantage PGF2α treatment is that it is luteolytic nature and induces oestrus in cows; the oestrus facilitates expulsion of bacteria and the products of inflammation and possible improvement in uterine defence mechanism (Kasimanickam et al. 2005). In a separate chapter, recent developments in therapeutic management of uterine infection in bovines are discussed.

10.11 Preventive Measures

Traditionally, routine herd health programs for reproduction have concentrated on repeated gynaecological examination to find status of uterus in relation to postpartum uterine infection and associated complications in reproductive cyclicity. If the problem is recognized, treatment is started to correct the disease. Essentially, the traditional approach to reproductive herd health is mostly concentrated in the animals that have been already affected by uterine infection and not aimed to prevention and early identification of development of reproductive problem. Is this economically good to optimize reproduction? Does one need to palpate every cow to identify the problematic cows? What percent of the cows in the herd have improved reproduction by these approaches? At present, availability of quantitative data on this aspect is very much limited. Therefore, it is pertinent to formulate strategies to identify the animals at the risk of developing uterine infection at an early stage and to apply strategic management to bring them back to normalcy so that postpartum fertility is not compromised.

To reduce the risk of developing postpartum uterine infection, all the three components of the disease triangle, i.e. pathogens, the environment, and animal, should be given due importance. The major reasons for an individual animal to be at risk of developing postpartum uterine infection are Pre-partum illness, Peri-partum complications, and Postpartum disorders (**3P-s**). Effective care and management offered during the "transition period" would keep the **3P-s** in low level so that the postpartum reproduction of the animal is not affected.

A 3P management strategy is proposed for preventing the uterine infection and managing after its occurrence.

3P management strategy for reducing uterine infection
1. Pre-partum feeding and health care
2. Peri-partum reproductive care and
3. Post-partum follow up

10.11.1 Pre-Partum Feeding and Health Care

To minimize the stress during pregnancy and calving, it is important that the nutritional requirements of the cows are fulfilled during the entire dry period with special care to vitamins, minerals, protein, and energy. Adequate care should be taken to avoid the weight loss in cows during the dry period. Satisfactory levels of minerals and vitamins must be there in tissues before calving and during postpartum to maintain the uterine health, but over-conditioning of cows should be avoided. Over-conditioning during pre-partum period makes the cows prone to infections, as over-conditioned cows may display poor tonicity of uterine muscles and experience more incidence of dystocia and earlier exhaustion during calving. In contrast, under-conditioned cows are prone to infection than cows in optimal condition. The body condition scoring (BCS) system is a valuable method for assessing reserves of body energy and nutritional status in dairy cows. The extent of loss in body condition and successive recovery during the calving to early lactation indicates the alterations in energy balance. Notable loss in body condition from dry to near calving culminates in more incidence of postpartum reproductive and metabolic disorders.

10.11.2 Peri-Partum Care and Management

Commonly, difficulty in parturition includes dystocia due to different factors including uterine inertia. Proper attention should be given to the cows with these problems, so that minimal harm happens to the reproductive organs. Retention of foetal membranes (RFM) poses additional risk for development of postpartum complications. Cows with RFM are prone to various peri-partum diseases that consist of, but not only limited to, ketosis, metritis, and mastitis, and reduce the disease resistance and milk yield. The RFM leads to reproductive problems such as postpartum metritis and an increase in the service period, calving interval, and calving to conception interval and days open. A vast repository of preventive and therapeutic regimes has been reported for RFM with variable efficacy. Clinical trials on intrauterine antibiotics for minimizing RFM induced metritis are confusing rather than clarifying. Prophylactic supplementation of vitamins and minerals during prepartum and use of hormones (like oxytocin, $PGF_2\alpha$) and ecbolics (like ergometrine) are also reported to be effective. Manual removal of foetal membranes is not advocated, but still widely practiced.

Periparturient disorders are not completely independent, but they are intricately interconnected disorders. Cow that had an episode of milk fever is 4 times highly prone for RFM and 2.3 times more prone for left side displacement of abomasum. Being affected with RFM increases the probability of ketosis incidence by 16.4 times (Correa et al. 1993). To attain good postpartum fertility, it is essential to keep the prevalence of metabolic disease within acceptable levels, as they can amplify the risk of postpartum metritis. The principal metabolic problems like the disorders linked to metabolism of energy (fatty liver, acute, subacute ruminal acidosis and ketosis) and disorders linked to metabolism of minerals (milk fever, udder edema, subclinical

Table 10.2 Targets for metabolic disorders in dairy cattle

Disorder	Target incidence rate
Milk fever	0–5%
Downer cow syndrome	<10%
Ketosis	0–5%
Abomasal displacement	0–5%
Retention of foetal membranes	<10%
Lameness	<15%

hypocalcaemia) should be kept in control. Among the disorders of metabolism, milk fever is a tricky periparturient disorder as it is associated with the processes of eight other periparturient diseases and its adverse effect on dry matter intake postpartum. Table 10.2 gives an indication on the standards of the incidence of metabolic diseases.

The management of transition cow must emphasize on preventing the adverse consequences of insufficient nutrition (protein imbalance, NEBAL, micro-elements, mineral insufficiency, and vitamin), enhancing BCS, and reducing negative effects of NEBAL, to permit a cow's reaction for the treatment.

10.11.3 Postpartum Follow-Up

Preventing serious postpartum disease conditions and ensuring good health are vital in the management of reproductive health. In majority of instances, the infection is not diagnosed until it becomes clinical that results in spending more time for cure and more money for therapy. Therefore, the postpartum follow-up should emphasize on earlier diagnosis and the protocol for postpartum follow-up should focus on early disease detection by repeated observation of animals. Monitoring of rectal temperature for the first 10 days postpartum could aid in identification of the problematic cows. Based on the findings of visual assessment (alert or depressed, dull) and rectal temperature monitoring, subsequent evaluation procedures are to be determined (rectal/vaginal). Based on the result of each assessment criteria, a set of procedure should be made for therapeutic use. Monitoring cow behaviour and body temperature is the currently used method for identification of ensuing metritis in cows. Systemic antibiotic is desirable when the body temperature exceeds 39.5 °C. Long-acting antibiotics were found efficient and non-steroidal anti-inflammatory drugs can be used in combination with the antibiotics.

10.12 Perspective and Prospective

Postpartum uterine diseases are common disorders of lactating dairy cows that negatively impact production and reproduction performance, thus diminishing profitability and sustainability of dairy operations. Despite damaging uterus, uterine

infections inhibit GnRH from hypothalamus and LH from pituitary, besides locally impacting the ovarian functions. In spite of the fact that the losses associated with uterine infection are huge, regrettably, the progress made toward prevention or control of uterine infections is very limited. Good knowledge about etiology, pathogenesis, improved diagnosis, therapy, and control measures is important to downsize the incidence of uterine infection in dairy cattle, which will be of economic and practical importance.

- Although precise estimates on the losses due to postpartum uterine infections are available in few developed countries, in developing countries such information are very limited. Developing such information is very much essential to underscore the importance of uterine infection under diverse production systems.
- Incidence of uterine disease is higher in cattle than the other species of domestic animals, indicating the possible faults or lacunae in husbandry of cow or a fundamental problem with some cow breeds (Sheldon et al. 2008). Therefore, substantial efforts are required to study the predisposing factors of uterine infections and the mechanisms of how the uterus detects infection, responds to microbes, and how the pathogen alters the normal uterine functions.
- The risk factors for uterine infection, which may vary with species, breed, and production system, are the basic requirements to evolve suitable preventive management strategies. Once the risk factors are understood, a standard "transition cow management" protocol can be developed for minimizing the negative energy balance, which makes the animal susceptible to development of postpartum uterine infection.
- Acquaintance with the molecular background of uterine infection will help in developing innovative methods to treat infertility associated with the condition. The postpartum uterine defense mechanism needs to be understood thoroughly. Knowing the balance between infection and immunity in the genital tract of female is the first and foremost to design suitable therapeutic and preventive strategies.
- Changing epidemiology of uterine infection is also a concern. In dairy cows, the diversity of uterine microbial composition is barely understood, although the evolving picture has shown to be highly complex (Santos and Bicalho 2012; Onnureddy et al. 2013). Therefore, discerning the ecology and complexity of microorganisms in the postpartum uterus is vital for devising control strategies for uterine infection.
- Interlinkage between the uterine infection, environment, reproduction, and immunity should be well-understood so that improved control and treatment strategies can be evolved.
- Although research on identifying pre-partum "bio-markers" for early detection of the cows at risk of developing postpartum metritis has been initiated very recently, we are still in infancy at this moment. Some preliminary experiments indicate that, owing to the changes in the tubular genitalia, some of the proteins present in uterine fluid might be specific or their levels might vary with subclinical uterine infection. Recent developments in proteomics offer a great scope for

identification, characterization, and quantification of all such proteins so that on-spot kits can be developed. Once "on-spot" diagnostic kits to identify the cows at risk of developing metritis at an early stage are developed, effective management strategies can be applied to prevent postpartum uterine infection and related consequences.

- Since there is a growing concern over the antibiotic resistance and its residue in milk, alternate therapies for treatment of uterine infection can be explored. Although information on intrauterine administration of LPS, oyster glycogen, neem oil, colostrum whey, etc. are available to a limited extent, the success rate and subsequent fertility need to be studied thoroughly especially under field conditions.

- The incidence of uterine infection can be downsized based upon adoption of preventive and control strategies including proper maintenance of animals, good breeding hygiene, proper care to transition cows, thorough nutritional programs, equipping with dry and clean housing areas, and appropriate recognition and treatment of cows affected with clinical and subclinical uterine infection. A robust extension mechanism to disseminate these strategies to the farmers can help in reducing the losses associated with uterine infection and associated consequences.

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Therapeutic Management of Postpartum Uterine Infections in Bovines

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Abstract

Uterine infections in dairy animals are significant problems during postpartum period. Majority of the animals get exposed to bacterial contaminations during calving process and up to 40% of the animals suffer from uterine infection which is classified as several types such as metritis or endometritis as per severity of clinical manifestations. Abnormal calving, calving assistance, negative energy balance, and compromised immunity are some of the important predisposing factors for uterine infections. Regular postpartum health check-up program will support accurate diagnosis and development of preventive or therapeutic measures. Individual or combinations of antibiotics and hormonal therapy are common protocol for management of uterine infections. Clinical studies on systemic and intrauterine administration of antibiotics often give conflict results and antimicrobials' use in food animals is believed to be an important driving force for antimicrobials-resistant pathogens' development. Therefore, alternatives to current therapeutic approach using natural or recombinant immunomodulatory products such as lipopolysaccharides, granulocytes macrophase colony stimulating factor, and herbal plants are recently explored and encouraging results are found. This chapter discusses about cause and consequence of uterine infections and various therapeutic approaches including antimicrobials, hormonal, and alternative therapies for uterine infections in dairy cattle.

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11.1 Introduction

Postpartum uterine infections are one of the most important reasons for sub-fertility or infertility in dairy animals. Uterine infections are classified into various forms based on their clinical manifestations and days in milk (DIM), of which metritis and endometritis are the major postpartum clinical conditions. Metritis is characterized by an enlarged uterus, containing a fetid odour and watery red-brown fluid to purulent discharge within 21 DIM with systemic involvement (Sheldon and Dobson 2004). Clinical endometritis is defined as the presence of a purulent or mucopurulent vaginal discharge during 21 days or after 26 days of postpartum period. The lactational incidence of metritis, including retained placenta, was reported about 21–40% in the USA and in other countries (Zwald et al. 2004; Haimerl and Heuwieser 2014). The incidence of clinical endometritis alone is reported between 10 and 20% (LeBlanc et al. 2002a) and the overall prevalence of uterine infections is reported up to 50% during postpartum period, in which incidence of metritis (10–20%), clinical endometritis (15%), and subclinical endometritis (15%) are common in cows (Lewis 1997; Sheldon and Dobson 2004; LeBlanc et al. 2011). The incidence and severity of uterine infections decrease with postpartum period. Sheldon and Dobson (2004) reported that up to 40% of the animals have uterine infection during initial two weeks postpartum, of which 10–15% showed persisted infections for at least another three weeks. Besides, 30–35% of cows suffer with subclinical endometritis between 4 and 9 weeks postpartum period (LeBlanc 2008). Unlike clinical endometeritis, subclinical form needs cytological techniques for its diagnosis, which is often not routinely practiced at farm level. Subclinical endometritis is also reported as one of the etiological factors for repeat breeder syndrome and its incidence was found to be 12–94% (Salasel et al. 2010) in India. Rantibioticn and Bawa (1977) found high prevalence (39%) of postpartum infections in buffaloes. Incidence of clinical metritis in crossbred cattle was reported up to 30% in India (Pal 2003). The major option for treatment or prevention of uterine infections includes the use of antibiotics or hormonal preparations. However, due to public health concerns of antimicrobials and synthetic chemicals' use in food animals, pharmaceutics other than synthetic antibiotics has been the focus of research in recent times. Growing concept of phyto-pharmaceuticals' application in food animal practices is an evidence for its future potential.

11.2 Uterine Diseases: Causes and Consequences

Postpartum uterine infections are commonly associated with mixed bacterial group. Among the various organisms, *E. coli* is believed to be an important pathogen in the first week of postpartum and it also involves in synergistic infection with other potential pathogens like *A. pyogenes* in 2–3 weeks postpartum (Dohmen et al. 1995; Williams et al. 2005). Bacterial pathogens have been classified as recognized, potential, and opportunistic uterine pathogens (Williams et al. 2007; Table 11.1).

Factors that contribute to higher rate of uterine infections include nutrition and energy status, environment, improper calving assistance, postpartum infusions, inaccurate heat detection, and uterine pathogens (O'Connor 1992). Calving assistance, twin births, and retention of foetal membrane (RFM) are important reasons for uterine infection (Bell and Roberts 2007). Opsomer and de Kruif (2009) reported the possible interaction between different postpartum diseases with risk factors in high yielding dairy cows and indicated that RFM followed by dystocia and still birth are the most important factors for metritis. Higher parity, abnormal calving (e.g. dystocia, abortion, and dead calf) and short gestation period were found to be important risk factors for RFM in Indian dairy animals. About 40–58% of the abnormal calving and only about 8–20% of the normal calving were associated with RFM in Indian dairy animals. The prevalence of RFM is also more during summer season and calves with lesser body weight in buffaloes. About 55–77% of the RFM-affected animals developed metritis and only 2–3% of the cows with normal expulsion of placenta developed metritis, indicating that RFM is an important risk factor of uterine infection in Indian dairy animals (Kumari et al. 2015; Fig. 11.1). Negative energy balance during transition period decreases the immune response of the animals and favors the development of uterine infections. Lactating cows that experienced negative energy balance and compromised their immune status during transition period were eventually diagnosed as clinical endometritis during later postpartum period (Manimaran et al. 2019a; Fig. 11.2). Upregulation of inflammatory genes under negative energy balance could be one of the reasons for alteration of immunity in these cows (Manimaran et al. 2019b; Fig. 11.3) and other studies also indicated the relationship between feed intake, negative energy balance, and uterine infections during early postpartum period (Dubuc et al. 2010).

Table 11.1 Classification of uterine pathogens

Recognized pathogens	Potential pathogens	Opportunist contaminants
Arcanobacterium pyogenes	*Bacillus licheniformis*	*Clostridium perfringens*
Prevotella melaninogenica	*Enterococcus faecalis*	*Klebsiella pneumoniae*
Escherichia coli	*Mannhiemia haemolytica*	*Micrococcus species*
Fusobacterium necrophorum	*Pasteurella multocida*	*Providencia stuartii*
Proteus sp.	*Peptostreptococcus* sp.	Coagulase negative *Staph.* sp
	S. aureus	Alpha-haemoltyic *Streptococci*
	Non-haemolytic *Streptococci*	*Streptococcus acidominimus*
		Aspergillus sp.
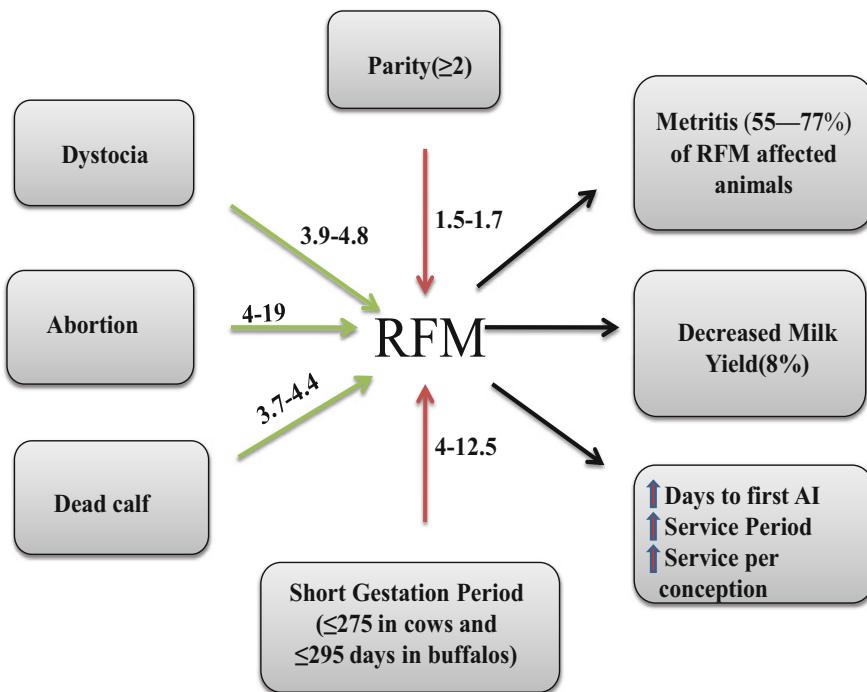
Fig. 11.1 Relationship of calving disorders, parity, and gestation period with metritis and postpartum performance with risk factors

Inflammatory mediators and microbial products (e.g. cytokines and LPS, respectively) reach the ovary and affect the ovarian function (Gilbert 2019).

Conception failure and subsequent culling are the major consequences of uterine infections. Globally, the reproductive problems are the leading causes of involuntary culling. Mohammadi and Sedighi (2009) reported that infertility was the single most reason for involuntary culling (35%), regardless of the age of the animal compared to other reasons like digestive tract problems (22%), mastitis (10%), and lameness (8%) in Iranian commercial dairy farms. It has been reported that the culling rate is 26% in the USA (USDA/NAHMS 2007), 37% in the UK, and up to 32% in Sweden due to fertility-related problems in cows. Low reproductive performance was also the most common culling reason in China and Brazil dairy farms (Wu et al. 2012; Cruz et al. 2011).

Mateus et al. (2002) reported that puerperal uterine infection significantly delayed uterine involution process and caused prolonged anoestrus and luteal phase of cycling cows. Opsomer et al. (2000) reported that cows with clinical endometritis were 4.5 times more likely to have delayed resumption of ovarian cyclicity and 4.4 times more likely to have prolonged postpartum luteal phases than healthy cows. The uterine infections in dairy cows not only affect the uterine tissue, but also do alter the entire hormonal milieu of the whole animal particularly the
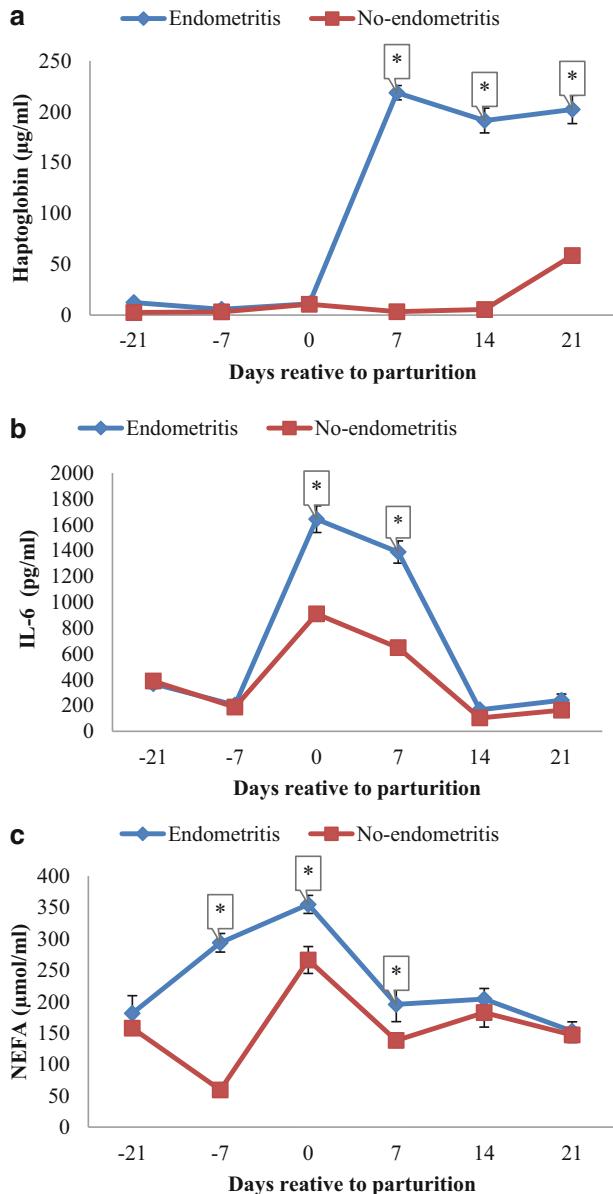
**Fig. 11.2** Changes of plasma concentration of haptaglobin (**a**), Interleukin-6 (**b**), and non-esterified fatty acids (**c**) during transition period in cows which eventually developed clinical endometritis ($n = 8$) and remained no-endometritis ($n = 8$). Lines (mean ± SE) bearing asterisk differ significantly ($P < 0.05$) between the groups at each sampling day intervals
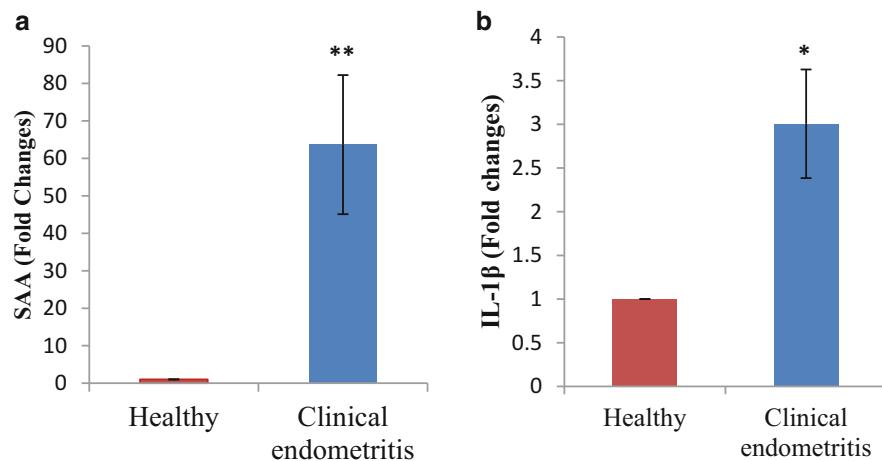
Fig. 11.3 mRNA expression of serum amyloid A (SAA) (**a**) and IL-1β (**b**) genes in the uterus of healthy and endometritis-affected cows. Values are means ± SE of three independent experiments. $*P < 0.05$, $**P < 0.001$

hypothalamic-pituitary-gonadal axis and the gonadotrophins secretion (Williams et al. 2008). Uterine infection also disrupts the ovarian follicle growth and function, with smaller and less steroidogenic ovarian follicles. Further, uterine infections also reduce oocyte quality, with increased rates of meiotic arrest and germinal vesicle breakdown failure (Bromfield and Sheldon 2011). The oocyte development from primordial follicle stage to ovulatory stage of a cumulus-oocyte complex takes about 120 days. On other hand, the cows are usually inseminated during 60 to 120 days postpartum period. Therefore, if the animal suffers with uterine disease during postpartum period, the ovulated oocytes may get exposed to pathogenic molecules and inflammatory mediators and produce long-lasting effects (Sheldon and Owens 2017). Fourichon et al. (2000) found that endometritis increased the service period (days open) and reduced pregnancy rate. The worst scenario is that even cows that were treated successfully for clinical endometritis had 20% lower conception rate and 3% of the animals remained infertile and were culled (Sheldon et al. 2009).

Cost associated with treatment and consequent milk discarding or residue violations in food of animal origin also cause considerable economic loss (Fourichon et al. 2000; LeBlanc 2008). Besides fertility, uterine infection also has an impact on milk production. Dubuc et al. (2011a) found 3.7 kg loss of milk production per day in multiparous cows due to metritis. When considering reproductive parameters alone, reproductive inefficiency beyond 100 days postpartum results in an estimated loss of $2.50 to $3.00 per cow per day, while overall loss due to reproductive inefficiency has been estimated to be $5.40 per cow per day (Plaizier et al. 1997). With approximately 100 dairy cows with 10% incidence of uterine infections, the cost of uterine infections/cow/month would be about $750–$1620. Therefore,

prevention or reduction of uterine infections load and improvement of uterine defense mechanism are most important for successful postpartum reproduction management.

11.3   Diagnosis of Uterine Infections

Accurate diagnosis of uterine infections is important pre-requisite to discrete a true treatment effect. Traditionally, uterine infections are diagnosed based on the nature of uterine secretion and systemic responses against infections like fever, reduced feed intake, etc. Recently, Sheldon et al. (2006, 2009) defined the postpartum inflammation of the uterus into puerperal metritis, clinical metritis, and clinical and subclinical endometritis based on abnormal uterus size, discharge, systemic signs, DIM, and other criteria. Subclinical endometritis is diagnosed by the percentage of neutrophils in postpartum uterine cytology samples (>18% during 21–33 days or > 10% during 34–47 DIM). Likewise, many researchers have proposed definition and classification of uterine infections (Dubuc et al. 2010). Apart from clinical signs, bacteriological and histo-pathological examinations of uterine samples through biopsy, uterine lavage, swab, and cytobrush techniques are the other diagnostic methods used in postpartum animals. There are two cytological techniques commonly used for the diagnosis of subclinical endometritis (Kasimanickam et al. 2005); the cytobrush method and the uterine flushing/lavage method. In cytobrush method, cytobrush being passed through the cervix and gently rotated against the endometrium and then cellular material is rolled on a slide for microscopic evaluation of PMN cells (Kasimanickam et al. 2004; Barlund et al. 2008). In uterine flushing method, the uterine lumen is flushed with a small volume of saline solution, which is recovered and examined by microscopy for the proportion of PMN cells (Gilbert et al. 2005). Kasimanickam et al. (2005) reported that cytobrush technique is a consistent and reliable method for obtaining endometrial samples for cytologic examination from postpartum cows. The current limitations in these methods are lack of consistent threshold level of PMN to define the disease condition during different time intervals of postpartum period.

11.4   Bacterial Isolation and Antimicrobial Sensitivity Assay in Uterine Infected Animals

Among the organisms isolated from postpartum uterus, *Arcanobacterium pyogenes* and *E. coli* were considered as major uterine pathogens (Sheldon et al. 2004; Williams et al. 2005). Malinowski et al. (2011) found *A. pyogenes* strains were most sensitive to amoxycillin/clavulanic acid (97%), bacitracin (97%), ceftiofur (96%), and cephapirin (78%) drugs and *E. coli* was most sensitive to norfloxacin (98%), marbofloxacin (96%), gentamicin (88%), amoxicillin/clavulanic acid (81%), and ceftiofur (73%). They found that *A. pyogenes* and *E. coli* were the most resistant to oxytetracycline (64% and 31%, respectively), which was also reported by other

workers (Cohen et al. 1995, 1996). Bekena et al. (1994) isolated bacterial flora in postpartum cows with RFM and found mixed infections were the most frequent with *A. pyogenes* together with gram-negative obligate anaerobic bacteria, especially *Bacteroides levii/spp.* and *Fusobacterium necrophorum,* and suggested synergistic role between these organisms. Westermann et al. (2010) isolated 34% and 10% of *A. pyogenes* and *E. coli,* respectively, from clinical endometritis cows through uterine swab during 21–28 days postpartum period. They compared the proportion of false positive diagnosis of clinical endometritis by vaginoscopy method and found 17% and 29% of the cows diagnosed as positive by vaginoscopic method were found to be negative by both bacteriological and uterine cytology methods. In contrast, percent of PMN cells was better predictor of reproductive performance than either intrauterine bacteriology or gross vaginal inflammation score as cows with highest number of PMN at both 28 and 42 days of postpartum had lower pregnancy rates, more days open, and lower milk yield than those in the lower PMN (%) categories (McDougall et al. 2011).

11.5   Therapeutic Management of Uterine Infections

Treatment of uterine infections is generally executed through intrauterine (i.u.) or systemic administration of antibiotics. Intrauterine administration of antiseptic solutions and systemic administration of single or combined hormonal preparation such as PGF2α and GnRH, etc., are also common practice in reproduction management. The major objectives of using antimicrobials are reduction of bacterial contamination and inflammation. Hormones such as PGF2α are mainly used for induction of luteolysis and consequent induction of estrus with increased uterine contractility and clearance of the uterine cavity. Better innate immune response of uterine tissue during estrus period in absence of progesterone-mediated immunosuppressive effects is also an added advantage of inducing estrus with hormones. More production of mucus during estrus also provides better defence system. Cows suffering from mild form of uterine infections are generally treated with hormones and cows suffering with uterine infections with systemic clinical manifestation such as fever, reduced appetite, and more loss of body condition during postpartum period are treated with antibiotics and supportive therapies (e.g. steroidal and non-steroidal anti-inflammatory drugs, fluid therapies, supplements, etc.). Although several trials have been conducted to see the clinical efficacy of these drugs, the results are not consistent and therefore treatment protocol often varies among veterinarians (Lefebvre and Stock 2012). Studies indicated beneficial effects of immunomodulators such as hyperimmune serum, *E. coli* lipopolysaccharides (LPS), Granulocytes Macrophage Colony Stimulating Factor (GM-CSF), Leucotriene B$_4$ (LTB$_4$), and Oyster Glycogen (OG) when they are administered through local route (Sarkar et al. 2016). Intrauterine administration of LPS, GM-CSF, LTB$_4$, and OG increases the influx of neutrophils and clears the bacterial infections. Herbal plants have also shown better efficacy in reduction of bacterial load in uterus-infected animals.

Antimicrobials are commonly used for reproductive problems, though the proportionate quantity of consumption is very less. For example, among overall sales of various pharmaceutical formulations for food producing animals in European Union (EU) countries during 2016, intrauterine preparations accounted for negligible amounts (mg/Population Corrected Unit; PCU) of 0.3% compared to 41% of premixes, 12% oral powders and 37% oral solutions, and 9% were injectables (EMA 2018). We also reported more of hormonal use than antiseptic solution or antimicrobials use for treatment related to uterine infections. Among the various antibiotics, oxytetracycline, enrofloxacin, and gentamicin were most commonly used for uterine infection in Indian dairy animals (Manimaran et al. 2019c; Baishya 2021). It is observed that only 10% of the total antibiotics were used for reproduction-related problems under individual farmer production conditions in southern India. Systemic, rather local, route of administration for treatment of uterine infections and other postpartum reproductive complications could be a reason for lesser sales of intrauterine preparations. For examples, parenteral antimicrobial treatment is recommended for severe form of uterine infections with systemic signs such as acute puerperal metritis, clinical metritis, and clinical endometritis. Intrauterine or systemic administration of antibiotic is also often practiced in RFM-affected animals, though it is not primarily an infectious disease, but an important predisposing factor for uterine infections (Sheldon et al. 2008). Though accurate quantity of antimicrobials consumption for reproductive problems is not available, more incidences of RFM and uterine infections suggest that they are potential indicator of antimicrobials use in dairy animals. Among overall indications, udder health management (dry cow therapy for preventive and systemic or intra-mammary administration as a therapeutic purpose) is by far the most important reason for antimicrobial use in dairy animals (Pol and Ruegg 2007). The little attention towards antibiotics in animal reproduction could be due to the following: (1) most of the postpartum diseases occur during voluntary waiting period when the uterine size is more and not suitable for intrauterine (i.u.) therapy, (2) pharmacokinetic and pharmacodynamic studies of various antibiotics under the influences of various hormonal, energy, and immune status of the animals have not been studied, (3) poor detection of subclinical infections, (4) lack of herd health programme and thus the treatment is neglected at the earliest possible time, and (5) clinical nature of the disease due to major pathogens and its susceptibility to antibiotics are not clear.

11.6   Effects of Antibiotics on Uterine Infections

Intrauterine (local) therapy is aimed to achieve higher drug concentrations at the site of infection, i.e. endometrium, but little penetration occurs to deeper layers of the uterus or other genital tissues. Penicillin, cephalosporins (e.g. ceftiofur and cephapirin), oxytetracycline, gentamicin, sulfonamides, and nitrofurasone groups of antibiotics are commonly administered via i.u. route. However, the efficacy of a local antibiotics treatment is a controversial issue, as some authors found it to be beneficial, whereas others have found it to have no effect. Nak et al. (2011) reported

that penicillins, cephalosoprins (third generations), and combination of penicillins with oxytetracyclines or cloxacillin are commonly used antibiotics for treating bovine metritis. Besides antibiotics, antiseptic solutions such as diluted lugols iodine (2%) and povidone iodine (0.25–0.5%) were also commonly infused via i.u. route. However, irritant nature of antiseptic solution makes it unsuitable for inflamed uterine tissue.

Thurmond et al. (1993) found that routine infusion of antibiotics (procaine penicillin G or oxytetracycline) may not be efficacious in endometritis. Farca et al. (1997) found that combined infusion of sterile EDTA-Tris solution with antibiotics (oxytetracycline, enrofloxacin, lincomycin-spectinomycin, or amikacin) caused complete recovery of endometritis in cows, which failed to be cured clinically when they were treated with the same antibiotics previously. Several researchers compared the efficacy of different antibiotics (ceftiofur, penicillin, and tetracyclines) in toxic puerperal metritis and found no significant clinical efficacy (Smith et al. 1998; Drillich et al. 2001). Collectively, it suggests that antibiotic therapy is not efficacious and did not improve the postpartum performance of dairy animals.

In contrast, beneficial effects of intrauterine infusion of antibiotics were reported by various researchers in subclinical (Kasimanickam et al. 2005) and clinical (LeBlanc et al. 2002b) endometritis. There are several biological factors that affect the treatment outcome, including the postpartum interval at treatment and whether the cow has a palpable corpus luteum (CL) at the time of treatment (LeBlanc et al. 2002b). They found no benefit of treating endometritis before 4-week postpartum using cephapirin or prostaglandin F2α (PGF2α). They found that treatment with PGF2α during 20–26 DIM in cows suffering from endometritis that did not have a palpable CL was associated with significant reduction in pregnancy rate. Single or combined use of hormonal treatment is generally followed for fertility disorders like acyclicity, silent heat, and endometritis cases, but it is not always successful in all clinical conditions. For instances, PGF2α alone may be effective for inducing estrous in animals with palpable CL and PGF2α in combination with GnRH and have positive effects on fertility of cows with endometritis. However, endometritis with recognized uterine pathogens appears to be responsive to antibiotics, but is advised after 27th day of postpartum (Gundling et al. 2012). Mari et al. (2012) compared the clinical and bacteriological recovery after single intrauterine administration of formosulphatiazole and cephapirin antibiotics with placebo group in cows affected with clinical endometritis during early postpartum period and found significantly higher clinical (reduction of total clinical score from 5.9 to 1 Vs from 5.6 to 4.6) and bacteriological cure (75–80% Vs 27%) in antibiotics-treated compared to placebo group. E. coli is the most common bacterial pathogen isolated from acute puerperal metritis cases and it is inherently resistant to penicillin. But, most of the cows treated with penicillin showed acceptable fertility performances and thus penicillin is a good choice for treatment of acute puerperal metritis (Ordell et al. 2016).

Further, Feldman et al. (2005) also reported that increasing DIM significantly increased the clinical cure rate from 60% (treatment before 42 DIM) to 80% (treatment after 42 DIM) in antibiotic- and hormone-treated animals. Kaczmarowski

et al. (2004) found best elimination of infections (66%) in cows treated with the intrauterine antibiotic in combination with $PGF_{2\alpha}$. Collectively, it suggests that apart from bacterial infection, other factors also influence the treatment efficacy in the animals. For example, McNally et al. (2014) reported that disease and physiological states of dairy cows determined the response to progesterone-based synchronization. They found that cows with more disease or physiological problems had the lower estrous response and conception rates, while anestrus and healthy cows were good responders to progesterone-based synchronization. It revealed that cows with disease conditions were not ideal candidates for synchronization. Most of the researchers evaluated the effects of antimicrobial therapy in uterine infection through fertility parameters like days open and did not evaluate the subsequent uterine health or bacterial infection. Galvao et al. (2009) studied the effect of i.u. infusion of ceftiofur at 44 ± 3 DIM in cows suffering from clinical endometritis. They found that i.u. infusion of ceftiofur did not influence the prevalence of clinical and subclinical endometritis when compared to control animals. However, it reduced the prevalence of positive uterine culture in cows with clinical endometritis and reduced the overall prevalence of *A. pyogenes*. They also found that *A. pyogenes* and *E. coli* were predominantly associated with clinical endometritis and *A. pyogenes* was common in subclinical endometritis (Galvao et al. 2009). Dubuc et al. (2011b) reported that administration of ceftiofur reduced the incidence of metritis in cows treated soon after parturition (24 h) at high risk of uterine disease (if they had twins, dystocia or RFM). Armengol and Fraile (2015) reported that combined administration of amoxicillin through parenteral route and intrauterine infusion of oxytetracycline resulted in significant reduction of the number of days to first insemination and days to conception compared with the parenteral treatment alone in heifers. Further, the combined route of treatment significantly increased the percentage of pregnant animals at the first insemination and decreased the percentage of non-pregnant cows at ≥ 150 DIM when compared with the parenteral treatment. Piccinno et al. (2014) investigated the modulatory effect of three antibiotics (amoxicillin, enrofloxacin, and rifaximin) on *in vitro* contractility of the bovine uterine tissue in follicular and luteal phases. They found that in addition to antimicrobial effects, amoxicillin relaxes and the enrofloxacin increases the uterine contractility in both cycle phases.

Haimerl and Heuwieser (2014) reviewed systematically the available literature (Table 11.2) on the treatment of acute puerperal metritis and found that the majority of the studies ($n = 17$ out of 21) were administered ceftiofur followed by oxytetracycline for the treatment of acute puerperal metritis.

We identified the bacterial isolates from clinical endometritis-affected cows, performed *in vitro* antibiotic sensitivity test against *E. coli* and subsequently evaluated the sensitive antibiotics in clinical endometritis-affected cows (Manimaran et al. 2019c). We found that gentamicin is most sensitive and oxytetracycline intermediately sensitive drug (Fig. 11.4). Gentamicin, oxytetracycline, and povidone iodine were administered for 3 days through i.u. route in endometritis-affected cows and found that the oxytetracycline was found to be more suitable than gentamicin for treatment of clinical endometritis cows. However, oxytetracycline was not as

Table 11.2 Research findings on the antibiotic therapy of puerperal metritis in dairy cows

References	Findings
Smith et al. (1998)	Treatment of cows affected with toxic puerperal metritis with procaine penicillin G (22,000 IU/kg, i.m. for 5 d), oxytetracycline (6 g i.u. infusion on d 1, 3, and 5), and ceftiofur sodium (2.2 mg/kg, i.m. for 5 d) showed a favorable response with similar treatment efficacy
Risco and Hernandez (2003)	Systemic administration of ceftioufur hydrochloride in dairy cows affected with RFM is beneficial for prevention of metritis, but its effect on reproductive performance is not significantly different from estradiol cypionate or no treatment
Chenault et al. (2004)	Administration of ceftiofur hydrochloride (2.2 mg of ceftiofur, SC or IM, once daily for 5 days) was efficacious for treatment of acute metritis in dairy cows
Goshen and Shpigel (2006)	i.u. infusion of chlortetracycline prevents the detrimental effect of clinical metritis on reproductive performance in heifers and cows
Drillich et al. (2007)	Single administration of NSAID along with systemic antibiotic (ceftiofur) treatment of cows having acute puerperal metritis resulted in no beneficial effects on clinical cure or reproductive performance
McLaughlin et al. (2012)	Administration of two doses of ceftiofur crystalline-free acid sterile suspension (d 0 and 3; 6.6 mg /kg of body weight) for metritis-affected cows showed higher clinical cure rate than for saline-injected cows (74% vs. 55%)
Jeremejeva et al. (2012)	Treatment of acute puerperal metritis and clinical metritis-affected cows using combination of parenteral antibiotic and NSAID showed no beneficial effects, while a combination of parenteral antibiotic and PGF2α showed same fertility parameters as healthy cows
McLaughlin et al. (2013)	Treatment of cows with a single dose of ceftiofur crystalline-free acid sterile suspension within 24 hours after abnormal calving (dystocia, twins, abortion, RFM, or any combination) reduced the incidence of subsequent metritis (17–25%) in lactating dairy cows
Sannmann et al. (2013)	No significant differences in cure rates, milk yield, or serum haptoglobin concentrations on DIM 2, 5, and 10 and subsequent uterine health (DIM 21–27) between treated and untreated cows
Lima et al. (2014)	Efficacy of ampicillin (11 mg/kg) or ceftiofur (2.2 mg/kg) once daily for 5 days in cows suffered with metritis and puerperal metritis during first 12 DIM was evaluated. Clinical cure was faster in ampicillin than ceftiofur-treated cows, but on d 12 both treatments resulted in similar cure. Clinical cure was less for cows with puerperal metritis than for metritis cows. Despite differences in uterine health, pregnancy at the first insemination did not differ among treatments

sensitive as gentamicin in *in vitro* test; more first-service conception rate and lesser days open were observed in oxytetracycline-treated cows (Fig. 11.5). Presently, oxytetracycline is the only FDA-approved antibiotic for treatment of metritis in USA and prolonged withdrawal period of oxytetracycline among treated animals is a major concern. Pecsi (2007) also found better response in oxytetracycline-treated cows than other treatment groups (amoxicillin and gentamicin). Runciman et al. (2008) reported that single treatment through i.u. route using cephapirin during 7–28 days postpartum period in cows at risk of developing endometritis resulted
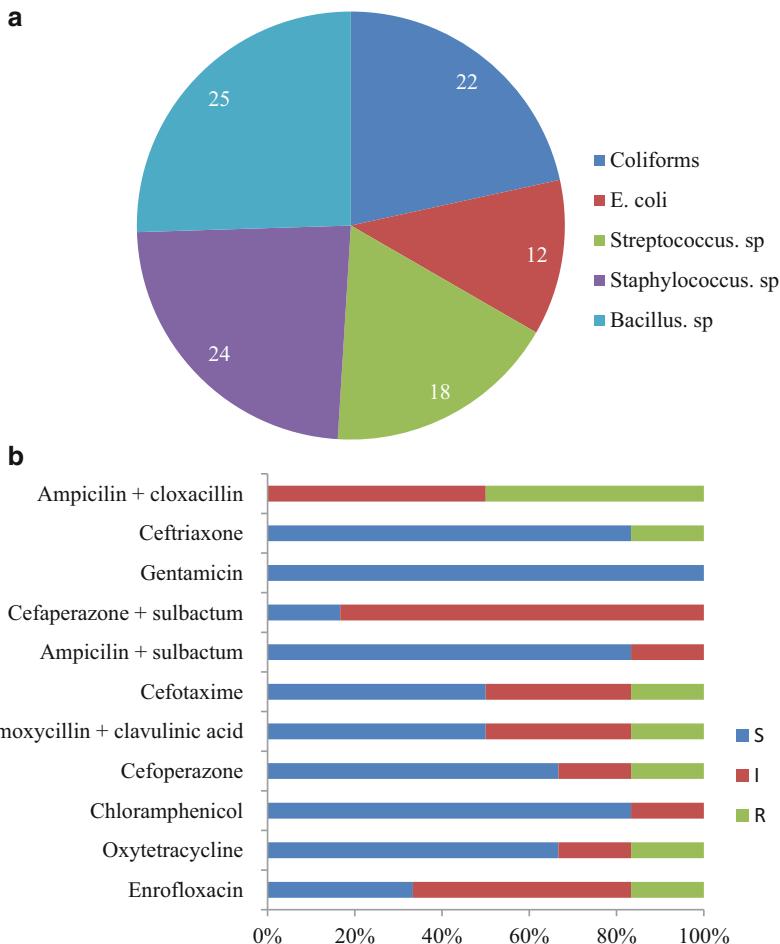
Fig. 11.4 Bacterial isolates (%) from postpartum endometritic cows (**a**) and antimicrobial susceptibility pattern of *E. coli* ($n = 8$) using disk diffusion method (**b**). *S*: sensitive, *I* intermediate, *R* resistant

in improved proportions of first-service conception and proportion of cows pregnant within 6 weeks of insemination with reduced service period. They also found better treatment effect in vaginal discharge positive cows and indicted calving to treatment interval and vaginal discharge status are important criteria to predict treatment outcome in terms of reproductive performance. They also compared two diagnostic methods (metricheck device and vaginoscopy) and their relationship with i.u. cephapirin treatment's effect on reproductive performance and found treatment of cows diagnosed with a purulent or mucopurulent discharge using both the methods and treated with antibiotic improved reproductive performance (Runciman et al. 2009).
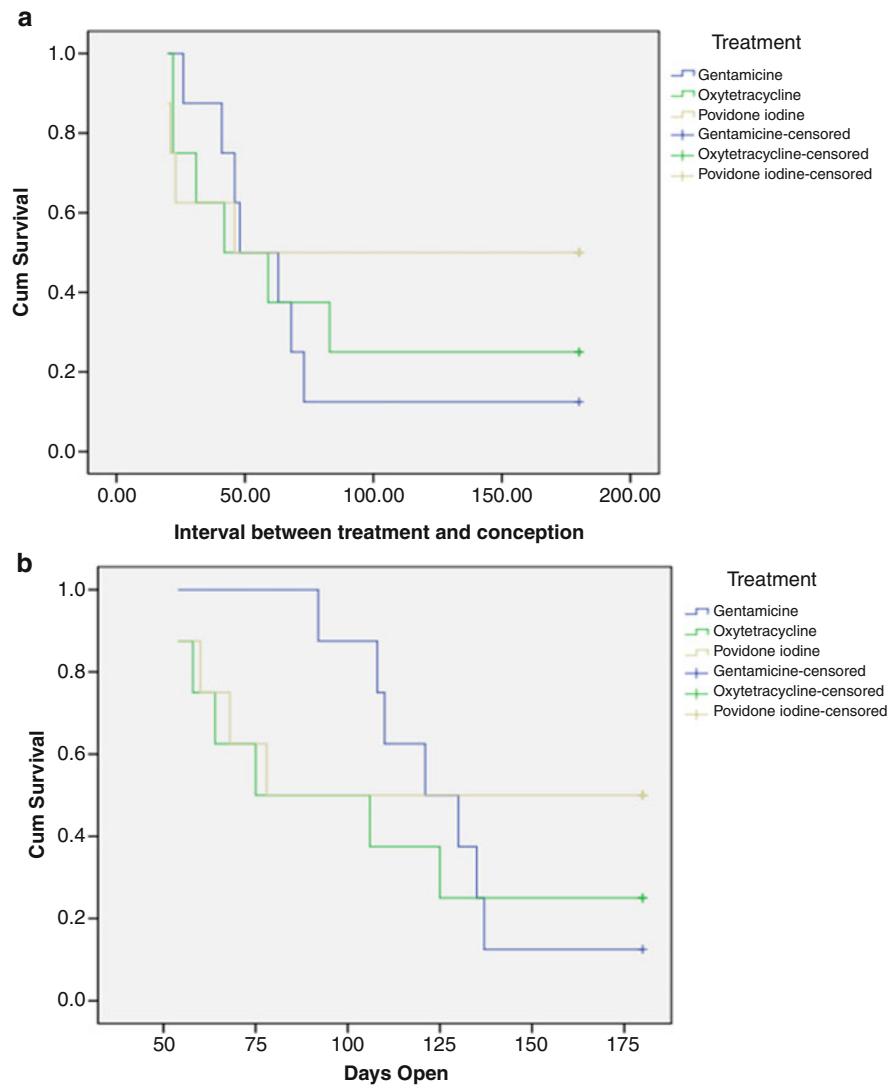
Fig. 11.5 Survival curve: chi squared values of log rank test for interval between treatment and conception (**a**) (0.76, $P = 0.68$) and days open (**b**) (1.003, $P = 0.61$) are different. Censored observation means that the animals were not conceived during follow-up period of 180 days postpartum

11.7 Effects of PGF2α on Uterine Infections

Investigation of the effect of PGF2α administration during 14 to 28 days postpartum period of cows that had risk of affecting subsequent fertility revealed no benefit in routine administration of PGF2α to dairy cows in that period when re-breeding commenced more than 70 days after calving (Glanvill and Dobson 1991). They also indicated that the presence of a CL did not influence the PGF2α treatment outcome. Later on, several studies reported no effect of PGF2α administration during 20–33 DIM (LeBlanc et al. 2002b) or 35–49 DIM (Dubuc et al. 2010) on subsequent reproductive performance. Feldman et al. (2005) reported that administration of PGF2α after 42 DIM resulted in significantly higher first-service conception rate with lower pregnancy index. They also observed that delayed uterine involution and isolation of *Arcanobacterium pyogenes* pathogens had overall negative impact on PGF2α treatment outcome. Antibiotic treatment provided better response in presence of recognized uterine pathogens. Non-specific bacteria and CL status had only minor effects on treatment outcome. PGF2α also stimulates the leucocytes functions and helps for early recovery from uterine infection.

11.8 Limitations in Evaluation of Treatment Outcome

Recent meta-analysis study revealed positive effect of ceftiofur for treatment of bovine acute metritis. However, advantages and disadvantages of ceftiofur than other antibiotics are not known. Comparative efficacy of different antibiotics with nonantibiotic therapy with respect to metritis prevalence is also not known due to limited comparative studies (Haimerl et al. 2018). Although quality of studies on evaluation of antibiotics and alternative treatment effects on uterine diseases are good enough, suboptimal level of consideration about (1) implementation of bacteriological examinations, evaluation of antimicrobial sensitivity among uterine pathogens, estimation of minimum inhibitory concentrations, (2) evaluation of reproductive performance as an outcome of treatment effect, (3) considerations of self-cure rates, and (4) considerations of critical issues such as antimicrobial resistance, prudent use of antibiotics, animal welfare, and cost-benefit ratios are some of the limitations among existing studies (Haimerl and Heuwieser 2014). They reported average self-cure rate of 37% (16–62%), while other researchers reported self-cure rate of 16 and 55% during 5 d and 14 d postpartum period, respectively (McLaughlin et al. 2012; Sannmann et al. 2013). Uniform times for re-examination with standard methods to determine the treatment success could improve homogeneity of trials and thus evaluation (Haimerl et al. 2018). In fact, lack of validated, consistent definition and outcome variables has been reported for endometritis (de Boer et al. 2014). Further, variations in pharmaceutical preparations and thus dosage and duration of same drug are also a cause of concerns for accurate evaluation of treatment efficacy. For example, ceftiofur has been most commonly used or evaluated drug against metritis with different preparations like ceftiofur -hydrochloride and crystalline-free acid formulations. The lack of untreated and unaffected controls, small sample size,

and assessment of treatment outcomes through clinical symptoms rather than repro-
ductive performance are some of the serious limitations in existing studies which
make difficult to assess the best therapeutic approach (Lefebvre and Stock 2012).

Although ceftiofur is very effective and has been recommended for uterine
infections, third- and fourth-generation cephalosporins including ceftiofur come
under critically important group of antibiotics. On the other hand, growing resistance
has been reported for these drugs in several bacteria in Germany. Further, presence
of same encoding genes for extended spectrum beta-lactamase in pathogen (*E. coli*)
isolated from poultry and infected human suggested for potential risk of resistant
development (WHO 2011). Besides, the judicious use of critically important
antibiotics, even drugs listed as not critical, may also pose threat to public health
due to transmission of antimicrobial resistance via mobile genetic element. There-
fore, Ozawa et al. (2012) encouraged for prudent use of all antibiotics and promote
the alternative therapies for health management including reproductive applications.
Preferential selection of antibiotics for uterine infections which are listed as less
critical was also proposed by Haimerl et al. (2018).

11.9   Alternative Therapies for Uterine Infections

Antimicrobial use is the single most important factor for development of antimicro-
bial resistance (AMR) in human and veterinary medicines (Grave et al. 1999) and
food animal production accounted for 70% of total antimicrobial consumption in EU
and USA, though such data lack in other developing countries. Although the AMR
development is a natural process, inappropriate use of antibiotics in both human
health and agricultural sector can speed up the development and spreading of AMR
pathogens. Besides, antimicrobials' use also cause detrimental effects on reproduc-
tive functions and efficacy data are also lacking in support of antimicrobials' use in
uterine infection (Pyörälä et al. 2014). Therefore, alternatives to antibiotics are focus
of research in recent times. For instance, based on the hypothesis of hypertonic sugar
solution-mediated inhibitory effect on bacterial growth (Chirife et al. 1983), several
researchers explored the possible use of hypertonic dextrose solution as an alterna-
tive to antibiotics in uterine-infected animals and found mixed response. Compari-
son of intrauterine infusion of hypertonic dextrose solution (50%) or systemic
administration of ceftiofur antibiotic revealed more pregnant animals in dextrose-
treated cows than ceftiofur or untreated control cows (Brick et al. 2012). Treatment
with dextrose also increased clinical cure; early resumption of cyclicity with more
pregnancy per AI in cows diagnosed with purulent vaginal discharge (Maquivar
et al. 2015). In contrast, Machado et al. (2015) reported that dextrose had a tendency
to cause a detrimental effect on clinical endometritis cure rate and had no beneficial
effects on reproductive performance. Intrauterine administration of *E. coli* LPS
(100 μg) significantly improved pregnancy rate in cows affected with endometritis
and repeat breeding, through improvement of innate immunity (Saini et al. 1999).
Intrauterine infusion of autologous plasma cured endometritis and improved con-
ception rate in clinical endometritis-affected cows (Methai 1999). Intrauterine

infusion of Oyster glycogen (OG) (500 mg) and LTB_4 (50 ml at concentration of 30 nmol/L) also showed clinical improvement in endometritis-affected cows with increased conception rate (Krishnan 2011).

Among various alternative therapies, herbal medicines are potential choice for efficient reproductive management of animals due to growing organic livestock farming, which does not permit any synthetic antibiotics to treat food producing animals. Application of indigenous knowledge of treating animal diseases and improving their productivity using medicinal herbal plants is known as ethno-veterinary medicine (EVM). Ethno-Veterinary Practice (EVP) is an age-old method of health management in various livestock species since their domestication. EVP using herbal medicines is mostly based on indigenous beliefs, technical knowledge, skills, methods, and practices pertaining to the health care of animals. Therefore, indigenous technical knowledge is often based on specific location and practiced by group of people for health management of animals. EVM is generally believed to be cost-effective with no or minimal side effects and easily available compared to several limitations of hormonal preparations such as affordability due to higher cost, accessibility particularly in villages, variable results, residual effect in food animals, etc.; all these make EVM a long-term alternative to modern medicines. Herbal medicines are used by >80% of the population, particularly in rural areas for primary health care, and maintain productivity in animals in Asian and African countries. Application of EVM for treatment of uterine infections of dairy cattle was also documented in ancient medicinal system and in recent literatures. Abbasi et al. (2013) reported that pathologies related to respiratory and reproductive disorders were most important veterinary conditions in which medicinal plants were used in similar ways of local human phytotherapy. Active research on plant bioactive compounds particularly using terpenes, lipids, saponin, and tannin containing plants to improve productive and reproductive efficiency in ruminant animals has been increased over the period time (Rochfort et al. 2008).

Several medicinal plants and their preparations were mentioned in Indian Materia medica for treatment of reproductive disorders. *Abroma augusta, Datura alba,* and *Musa sapientum* for dysmenorrhoea, *Amorphophallus sylvaticus, Aristolochia bracteata, Balsamodendran myrrah, Cardiospermum helicabum, Parmelia perlata, Rubia cordifolia, Sapindus trifoliatus, Salvadora persica, Semecarpus anacordium,* and *Sesamun indicum* for amenorrhoea, *Entada pursoetha, Saraca indica, Nigella sativa,* and *Pedalium murex* for uterine disorders, *Mytrus communis* and *Viola sp.* for uterine prolapse, *Pandanus odoratissimus, Plantgo sp.* and *Viburnum foetidum* for abortion and other postpartum complications such as postpartum haemorrhage are some of the documented herbal plants and their preparation in Indian materia medica (Nadkarni 1954). A combination of herbal plants such as *Lepidium sativum, Citrullus colocynthis, Plumbago Zeylanica, Gossypium herbaceum, Peganum harmala, Gloriosa superba, Rubia cordifolia, Aristolochia indica,* and *Aloe barbadensis* with other plants having ecbolic, uterine stimulant, antimicrobial, anti-inflammatory, analgesics, and muscular stimulant properties was prepared and marketed as bolus and liquid formulation for expulsion of retained placenta and subsequent uterine cleansing purpose, regulation of lochial discharge, and

expediting the uterine involution in dairy cattle and buffaloes (Exapar™ and Liq. AV/UTL/17™, M/s Ayurvet Limited, India) (Singal 1996; Walia et al. 2010). Similarly, several polyherbal preparations such as Prajana HS (Indian Herbs, Natural Remedies), Janova (Dabur, Ayurvet), Fertivet (Ar Ex Labs), Sajani (Sarabhai), Heat-Up (Century), HimROP (Himalaya), Uterotone (Cattle Remedies), and Heatraj (Rajan) are being commercially marketed in India for induction of ovarian activity in anoestrus animals and to facilitate the expulsion of retained fetal membranes, secretion of lochial discharge, uterine involution, and resumption of cyclic activity in farm animals.

11.9.1 Plants Used for Retained Placenta

Retained placenta is one of the important periparturient disorders in dairy cattle due to multiple etiological factors. Failures in separation and expulsion of foetal membrane within 8–12 hr. after calving are mainly due to hormone dysregulations that resulted in altered structural and immunological functions of bovine placentome during parturition (Attupuram et al. 2016). Peripartum immunosuppressant is also one of the important reasons for occurrence of RFM. The manual removal of placenta is still controversial and currently no effective treatment is available for treatments for RFM. Although systemic administration of antibiotics is shown to have beneficial effects through prevention of metritis after RFM, they failed to show long-term benefits in terms of fertility performances in cows suffering from RFM (Beagley et al. 2010). Huang et al. (2018) reported that administration of an herbal powder based on traditional Chinese veterinary medicine in Holstein dairy cows affected with RFM (0.5 g crude herb/kg bw, p.o. once daily for 1–3 days) enhanced the fertility (calving-to-first-AI interval, services per conception, days open, and percentage of cows pregnant within 180 days postpartum) than in ceftiofur-treated group. This beneficial clinical efficacy represents a potential of herbal medicines to improve the subsequent fertility of dairy cows. Cui et al. (2017) also reported that oral administration of the Chinese herbal powder in cows affected with RFM accelerated placenta expulsion and had superior clinical efficacy in metritis prevention compared to the systemic administration of ceftiofur antibiotic-treated cows during early postpartum period. Oral preparations from leaves of *Balanites aegyptica* Del., *T. Indica,* and *Ficus thonningii* Blume and parts of *Hibiscus sabdariffa* Linn, and *S. bicolor* Linn. Moench with external application of ash powder around vaginal area were traditionally practiced in ruminant animals in Nigeria to induce expulsion of placenta (Alwa et al. 2002). Lans et al. (2007) reported the practices of using *Hedera helix* and *Alchemilla vulgaris* herbal feeding for RFM in Canada. Similarly, feeding of several herbal preparations in traditional Chinese (Table 11.3) and Indian medicinal system has been reported to facilitate the expulsion of placenta.

Feeding of decoction prepared from 2 kg *Pennisetum americanum* grain, 100 g of *Trigonella foenum-graecum* seeds, 50 g of *Lepidium sativum*, 25 g of *Anethum graveolens,* and 500 g of joggery resulted in expulsion of placenta within 2–3 h.

Table 11.3 Patented Chinese herbal medicines' formulation for retained placenta treatment (Source: Zuo et al. 2016)

S. No.	Medicinal plants	Formulations	Application	Findings
1	Angelica, Talcum, Rehmannia root, Radix astragali, Tuckahoe, Peach kernel, Motherwort, Radix codonopsitis Safflower, Licorice	Decoction	Oral administration of one decoction per day	95% success after 12 h of administration
2	Angelica, Wallichii, Garden balsam, Rhizoma ligustici, Radix codonopsitis, Motherwort	Perfusion	Rectal perfusion of 150 ml once a day at 35–40 °C	98% cure rate
3	Herba Leonuri, Angelicae Sinensis Radix, Flos Carthami, Myrrha, Rhizoma Cyperi	Tincture	Oral administration of 0.45 g herb/kg BW/for one day	73% success within 72 h
4	Fructus meliae toosendan, Radix bupleuri, Semen litchi, Fennel Frankincense Notopterygium root	Decoction	0.4 g crude herb/kg BW/BID, fed directly or mixed with feed	95% recovery within 4–20 days
5	Garden balsam, Motherwort, Dried ginger Angelica, Peach kernel Safflower, Myrrh Rhizoma cyperi	Tincture	1 ml tincture/ kg BW orally, once or twice	84% cows expelled placenta within 48 h
6	Motherwort, Chinese angelica, Chuanxiong, Semen persicae, Dry ginger Licorice	Powder	Oral administration (250 g per cow) with warm water, for 3–5 times at 6 days interval	Not available
7	Dang Hong Fu	Infusion	40 ml extracts or 40 g herbs, i.u. infusion	83% recovery

Feeding of decoction prepared from bamboo leaves, bark, and paddy husk was also practiced for RFM treatment. Preparations from leaves and twigs of Zyzyphus mauritiana and Tassels of maize cobs were also fed to animals after calving for smooth expulsion of placenta (Perumal et al. 2013). Several plants such as Ficus benghalensis, Legernaria vulgaris, leaves of jingara, root bark of Caesalpinia bonducella (kanarej), cotton shells and roots of cotton plant, and sugarcane leaf juice were used for treatment of RFM (Perumal et al. 2013). The root bark of *Caesalpinia bonducella* was shown to increase the contractile force of pregnant rat myometrium in *in vitro* organ bath studies (Ali Sagar et al. 2003). *Abrus precatorius, abutilon indicum, Anethum suva, and Ficus glomerata* are also used to remove placenta (Singh and Khan 1999). Kumar and Bharati (2013) reported that gum of *Acacia nilotica* Delile, leaf paste of *Basella alba*, whole plant of *Boerhavia diffusa*,

Oxalis corniculata, seed oil of *Brassica napus* (*Rape/*oilseed rape*/*rapa), dried flower of *Corchorus capsularis* (*Nalta jute*), root of *Ficus benghalensis* (*Bargad*), leaf of *Mimosa pudica* (*Sensitive plant, sleepy plant, or shy plant*), and whiskey of *Saccharum officinarum* (*Sugar cane*) are used to manage the RFM in animals in Uttar Pradesh (India).

11.9.2 Plants Used for Uterine Infections

Chinese herbal compositions of *Radix Salviae Miltiorrhizae, Radix Arnebiae (Radix Lithospermi), Borneolum Syntheticum,* and edible vegetable oil were patented for treating endometritis in dairy animals with cure rate of 91% (Patent No: CN1650950A, China). Compositions of *Herba Leonuri, Flos Carthami, the Radix Astragali, Radix Scutellariae, Caulis Akebiae, Rhizoma Chuanxiong, Flos Lonicerae, Fructus Forsythiae, Radix Et Rhizoma Rhei, Radix Codonopsis, the Radix Rehmanniae, Radix Angelicae Sinensis, Herba Taraxaci, Herba Epimedii,* and sweet were also patented for treating endometritis (Patent No: CN103239554B, China). Ali Sagar et al. (2003) reported that the immunomodulatory property of *Aristolochia indica* (Isharmur) can be used for preventing the uterine infection. The concoction prepared from powder of root or bark of the *Convolvulus microphyllus* was reported to cure uterine infection after feeding animals once a day for three days (Parmar 1999). The juice prepared from *Helicieres isoro* fruits was also reported for treatment of uterine infections in India (Tripathi and Sikarwar 2013). Herbs like blue cohosh roots (blue ginseng), *Lamium album* (white nettle or white dead nettle), and *Hydrastis canadensis* (goldenseal) have been used for treating endometritis in women. Treatment with 90 ml intrauterine infusion of comfrey extract for three days has been recommended for metritis in dairy animals and the same was patented in USA due to beneficial effect on endometrium without any irritation (Noorlander 1987). Verma et al. (2016) conducted field study to evaluate the Uterofix™ (M/s Ayurvet Limited, India), intrauterine liquid (25 ml for 3–5 days) containing herbs such as *Plumbago zeylanica*, *Azadirachta indica*, *Acacia catechu Curcuma longa,* and *Saraca indica* on endometritis, anestrous, metritis, and repeat breeding cows, which resulted in 90% recovery from these conditions and showed heat symptoms with clear discharge. Antibacterial and immunomodulatory properties of these herbs could be the reasons for such beneficial effects.

11.10 Conclusions

Therapeutic management of uterine infections is an important tool for successful reproductive management in dairy animals. Several systematic studies confirm the beneficial effects of cephapirin and ceftiofur drugs in clinical endometritis and clinical metritis cows, respectively. Lack of side effects or residue violation due to cephapirin is also an advantage for its usage. Effect of PGF2α is not clear in endometritis cows. Understanding the relationship of uterine pathogens, its extent

of inflammatory response (quantifiable through standard methods like uterine cytology, etc.), and its clinical manifestation (diagnosed by vaginal discharge) along with antibiotic sensitivity tests is the very basis for developing suitable treatment protocol for uterine infections. Understanding the role of several factors such as days in milk, parity, metabolic status, status of corpus luteum, drug formulation, sample size, randomization, with suitable criteria for diagnosis, and evaluation of treatment response is very important for judgment of clinical trials. Lack of systematic research under natural clinical conditions is one of the reasons for such limitations. Only systemic route administration during early postpartum to treat metritis or risky group of animals (e.g. cows with abortion, dystokia and RFM) and both systemic and local route of antibiotic administration after uterine involution may be appropriate way to check uterine infections. Selection of the most appropriate drug at its optimal dosage and duration is also important to cure an infection and reduce the resistant strains development. Combined use of hormones and antibiotics should also be explored to increase the elimination of uterine infections. With increasing public health concern about antimicrobial resistance, systematic studies with alternative herbal therapies would be long-term solution to manage uterine infections in dairy animals.

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Recent Developments in Bovine Semen Cryopreservation

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Abstract

The first-generation reproductive technology, artificial insemination is the tool that expedited the genetic progress in cattle and buffaloes during last few decades. The genetic improvement can be achieved only when superior quality bulls are used for artificial insemination. Even when the superior bulls are used for cryopreservation, their spermatozoa are also prone to many damages during cryopreservation. Cryopreservation is a damaging phenomenon, leading to significant reduction of motility (nearly about 50%) and fertilizing capacity of spermatozoa. The altered spermatozoa metabolism, ultra-structure, spermatozoa kinetics, cryocapacitation like modifications, increased protein tyrosine phosphorylation, production of reactive oxygen species (ROS), spermatozoa chromatin damage, mitochondrial damage, spermatozoa RNA, and protein alterations are reported by the recent studies with the help of evolution in spermatozoa analyses methods such as flow cytometer and different omics approaches. Even though the spermatozoa cryopreservation is now a standardized process and regularly performed in commercial scale, we have not attained complacence, since there is a scope to tailor and fine-tune to improve the cryosurvival of spermatozoa. In this chapter, we discussed the different cryodamages happening to the spermatozoa during cryopreservation, the role of extenders in protecting cryodamages, and recent developments in cryopreservation of bull semen.

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12.1 Introduction

Artificial insemination and cryopreservation have traversed a long evolutionary journey and become a time-tested biotechnological tool for genetic improvement. Advances in frozen semen technology have improved post-thaw semen quality, which is reflected in improved fertility with frozen-thawed semen over time (Foote 2003). With such improvement and optimized results, the transmission of superior genetic material by artificial insemination has become rapid and widespread, crossing geographical boundaries. The relationship between semen quality and fertility could not be fully defined, but it is largely accepted that the conception rate of frozen semen is less than fresh semen (Graham and Moce 2005). Cryopreservation leads to a reduction in motility by around 50% and significantly affects the fertilizing capacity of spermatozoa (Watson 2000). Thus in order to evolve an improved cryopreservation method with improved quality of the end product, it is required to understand the underlying principles and damages associated with cryopreservation of semen.

Cryopreservation is a damaging phenomenon (Bailey et al. 2003) to spermatozoa, and to develop an efficient method, understanding the cryodamage is of utmost importance. The changes are manifold, ranging from alteration in spermatozoa metabolism and ultra-structure, developing cryocapacitation like changes, protein tyrosine phosphorylation to the production of reactive oxygen species. All lead to sub-lethal injuries in cellular components and cell membrane, leading to reduced fertility. Since the serendipitous discovery of the cryoprotectant property of glycerol (Polge et al. 1949; Polge and Rowson 1952), several extenders were developed to counter these changes, and they are presently able to contend the damages to an acceptable level. Presently, Tris, along with egg yolk or milk, is used as a universally accepted base for an extender for semen cryopreservation of bovine semen (Batellier et al. 2001; Barbas and Mascarenhas 2009; Medeiros et al. 2002; Singh et al. 2012; Layek et al. 2016).

Though cryopreservation of bull semen has become a quite standardized process, the quest for improving the post-thaw performance is continuing and leading to further fine-tuning and new developments. The changes involve identifying an alternate candidate for cryoprotectant, alternate methods for cryopreservation, and inclusion of different additives in extender to minimize changes associated with cryopreservation.

The present chapter will thus look into the basic understanding of cryopreservation of bull semen, extenders in protecting cryo damage, recent developments in cryopreservation of bull semen, and future research needs.

12.2   What Changes During Cryopreservation?

The successful cryopreservation is based on many factors, like interactions between extenders, cryoprotectants, packaging method, freezing, thawing protocol, etc., apart from individual variations (Cotter et al. 2005; Andrabi 2007; Clulow et al. 2008). Loss of spermatozoa viability to some extent is unavoidable due to the procedures prior to freezing and also during the actual freezing process. Even the apparently viable spermatozoa after cryopreservation are found to be compromised in multiple functional parameters compared to their fresh counterparts.

Fertilizing ability of spermatozoa is highly dependent on spermatozoa motility, capability to generate energy via metabolism, ability to preserve normal plasma membrane conformation and integrity, and ability to maintain enzymes reserve, such as acrosin in the acrosome to allow the penetration into ova. The fertility of spermatozoa would be significantly affected if any of these functions were disturbed. The extreme risk is posed by the ice crystals formation, leading to the movement of water against osmotic gradients during cryopreservation. When the temperature reduces below freezing, the sample undergoes supercooling. As the temperature reduces beyond supercooling, extracellular ice crystal formation begins, thus uplifting the concentration of solutes in the surrounding medium. In contrast, water within the spermatozoa is slower to form ice crystal than the water in the surrounding medium. Subsequent exosmosis drives water out of the spermatozoa, particularly from the spermatozoa head, across the semi-permeable plasma membrane, and the spermatozoa become highly dehydrated (Watson 2000; Andrabi 2007). Alternatively, if the cooling rate is rapid, water has less time to come out of the spermatozoa, and hence large intracellular ice crystals form, resulting in physical damage to cell membranes and intracellular components. Nevertheless, the problems of solute concentration and dehydration are less apparent with rapid cooling.

The rate of temperature reduction has a notable effect on the rate of efflux of water from the spermatozoa; i.e. the slower cooling rate increases the duration of efflux and hence greater dehydration. Apparently, this does reduce the ice crystal formation causing substantial physical damage to the spermatozoa, but even bigger damage happens as a result of higher intracellular dehydration. A cryopreservation methodology should, therefore, aim at designing an optimal cooling rate that will afford a balance between all these factors.

In addition to ice crystal formation, other related factors which highly impact the fertilizing ability of spermatozoa are protein tyrosine phosphorylation, cryocapacitation like changes, formation of reactive oxygen species (ROS, such as superoxide anion, nitric oxide, and hydrogen peroxide), lipid scrambling, and spermatozoa plasma membrane damage.

12.3 Cryo-Injuries to Spermatozoa During Cryopreservation

The cryopreservation process includes the steps of reduction of temperature, dehydration of cells, freezing, and thawing. It disturbs the usual function and viability of spermatozoa in various ways, and the impacts are described. The effect of cryopreservation on spermatozoa is depicted in Fig. 12.1.

12.3.1 Changes in Spermatozoa Metabolism

The structural alterations produced in the post-thaw spermatozoa membrane are mainly connected to altered capabilities for energy sourcing. This would impact both metabolism and other spermatozoa functional attributes such as motility (Cerolini et al. 2001; Gillan et al. 2004; Dziekońska et al. 2009). Besides physical stress, cryopreservation of spermatozoa is also linked with oxidative stress (Mazur et al. 2000; Chatterjee et al. 2001). Spermatozoa are extremely susceptible to oxidative stress owing to their complete dependency on aerobic metabolism to
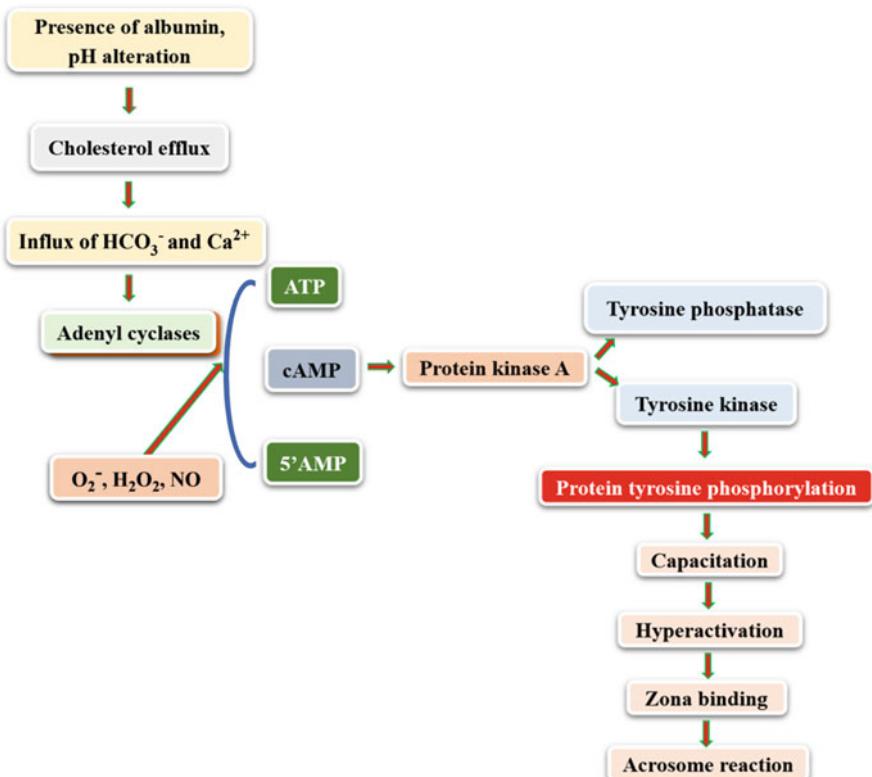

Fig. 12.1 The impact of cryopreservation on spermatozoa

meet ATP necessities. Commonly, the intracellular ATP concentration is decreased/ lost, and the AMP/ADP rate is increased by cryopreservation. Spermatozoa motility, the most commonly used parameter for assessing the quality of the ejaculate and post-thaw frozen semen quality, is highly reliant on mitochondrial function. The ATP produced in the inner mitochondrial membrane by oxidative phosphorylation is relocated to the microtubules to generate motility. Therefore, reduced spermatozoa motility after cryopreservation is believed to be mostly linked with mitochondrial damage (Ruiz-Pesini et al. 2001). In support of this hypothesis, many ultra-structural studies affirmed the occurrence of harmful effects of cryopreservation on spermatozoa organelles along with mitochondria (Nishijima et al. 2014).

Further, as spermatozoa are not able to produce antioxidants, higher oxidative stress, induced through multiple pathways, predisposes spermatozoa to oxidative stress during cryopreservation (Aitken and Baker 2004).

A slow reduction of metabolic activity of spermatozoa during cryopreservation could reduce the production of harmful metabolic by-products, which might impair spermatozoa function. However, metabolic activity reduced in this way may impact vital spermatozoa functions necessary for successful conception.

12.3.2 Changes in Spermatozoa Ultra-Structure

Plasma membrane of spermatozoa is highly sensitive to lower temperature, which is the principal site of damage during cryopreservation. Freezing induces membrane modification alterations in protein activity and consequently alters the permeability of water and solutes. This ends up in a considerable loss of viable spermatozoa (Bailey et al. 2003). The characteristics of membranes that impact their sensitivity include protein: phospholipid ratio, cholesterol: phospholipid ratio, non-bilayer-preferring lipids content, and degree of hydrocarbon chain saturation (Medeiros et al. 2002). The two foremost factors that affect fluidity are the relative concentrations of cholesterol and phospholipids, in which increased concentrations of phospholipids lead to high membrane fluidity. The key phospholipids of the spermatozoa plasma membrane are phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. Phosphatidylcholine and sphingomyelin are linked to the outer layer of the bilayer, while phosphatidylethanolamine is linked to the inner cytosolic layer. These affinities are not usually obvious except during the stress in the membrane (Quinn 1989). Therefore, the cold shock causes alterations to the distribution of the phospholipids across the bilayer, which causes altered membrane function (Bailey et al. 2003). Additionally, swelling of the acrosome due to cold shock specifies a loss of membrane integrity and inability to stretch.

Spermatozoa membrane proteins are also a key component in relation to sperm viability. The protein-lipid interactions are crucial for effective membrane function. It is vital to ensure proper moulding and distribution of proteins into the bilayer to eliminate membrane faults and pores. These communications are also required for the effective functioning of these proteins as enzymes, channels, or receptors for the exchange of ions like calcium ions. Spermatozoa alteration related to freezing is

linked to the transfer of proteins via the cell, which is controlled by the distribution of lipids in the membrane, altered response to induced capacitation, and the acrosome reaction of cryopreserved spermatozoa during fertilization (Guthrie and Welch 2005).

12.3.3 Changes in Spermatozoa Kinematics

Cryopreservation changes spermatozoa kinematics through irreversible changes in the mitochondria mid-piece and coiling of the tail. The evolution in spermatozoa analysis let us study the numerous structural and kinematic parameters of spermatozoa using a computer-assisted spermatozoa analyser (CASA). The kinetic parameters of bull spermatozoa assessed by CASA have a correlation with fertility. For instance, a higher positive correlation (ranging between 0.60 and 0.74) was reported between the VAP (average path velocity), VCL (curvilinear velocity), and VSL (straight line velocity) of spermatozoa and the fertility (Gillan et al. 2008; Kathiravan et al. 2008; Padrik et al. 2012; Shojaei et al. 2012). It was reported that total motility (TM %), rapid velocity (RV %), progressive motility (%), VAP (μm/ sec), VCL (μm/sec), VSL (μm/sec), STR (%), BCF (beat cross frequency, Hz), and LIN (linearity %) were significantly reduced after the freeze-thawing process (Ahmed et al. 2019). However, when the kinematics were evaluated at different stages of cryopreservation (i.e. on dilution, equilibration, post-thaw), all the parameters were significantly decreased after the equilibration stage of cryopreservation itself (Sundararaman et al. 2012).

12.3.4 Cryocapacitation Like Changes Due to Cryopreservation

Spermatozoa capacitation is an amalgamation of biochemical events that begins after spermatozoa enter the female reproductive tract. The spermatozoa capacitation mechanism is connected with intracellular ionic modifications and spermatozoa membrane (Pons-Rejraji et al. 2009). These changes include inactivation or removal of decapacitation factors by surface changes in spermatozoa like alterations in molecular structure, localization, lateral mobility of integral proteins and ionic deregulation displayed as increased internal Na^+, Ca^{2+}, and pH, generation of ROS, and an increased cAMP and protein tyrosine phosphorylation (Bailey et al. 2003; Bailey 2010).

Cryopreservation also brings several comparable changes to the spermatozoa, like calcium influx (Bailey et al. 2003). During cryopreservation, spermatozoa fail to appropriately moderate normal levels of internal calcium. Distorted lipid-protein associations and restructured membranes favour additional calcium influx during cryopreservation. Disturbance in the normal capacitation and acrosome reaction due to abnormal calcium ion concentrations would extremely compromise the fertilizing potential of post-thaw spermatozoa. An increased concentration of calcium in spermatozoa has been established during capacitation in many mammalian species,

including bovine and swine (Bailey et al. 2003; Tardif et al. 2001; Kumaresan et al. 2011). It can be said that these changes may occur during cryocapacitation also. It has been proved that the Ca^{2+} levels were more in cryopreserved than fresh semen, which further supports the theory.

Cryopreservation has been shown to cause the premature acrosome reaction in spermatozoa (Gadella et al. 2008). Acrosomal and plasma membrane vesiculations occur during spermatozoa death which is known as a false acrosomal reaction. The true acrosome reaction occurs before fertilization in the female reproductive tract, only in intact live spermatozoa (Yanagimachi 1994). Nevertheless, cryopreservation inducts acrosome reaction-like changes and makes the spermatozoa incapable of reaching the site of fertilization. Therefore, it is vital not only to analyse spermatozoa viability, but also to evaluate the intactness of acrosome concurrently.

The cryopreserved spermatozoa display capacitation-like modifications and appear in a partially capacitated stage. Cryocapacitation (capacitation-like effect) leads to reduced survivability of cryopreserved spermatozoa in the female reproductive tract, resulting in poor conception with frozen-thawed semen compared to fresh semen (Bailey et al. 2003; Watson 2000). The cryocapacitation has been proved by the presence of a higher proportion of chlortetracycline (CTC) fluorescent pattern "B" in frozen-thawed spermatozoa of bull (Collin et al. 2000), boar (Kaneto et al. 2002), equine (Thomas et al. 2006), and buffalo (Kathiravan et al. 2008). Normally, capacitation makes a destabilization state with which the spermatozoa acquires the fertilizing capability. However, if the spermatozoa fail to fertilize, they are vulnerable to membrane degeneration and spontaneous acrosomal reaction (Bailey et al. 2003). The process of cryopreservation makes a subpopulation of partially/fully capacitated and killed spermatozoa, which has a shortened lifespan in vivo, impairing fertilization potential.

12.3.5 Generation of Reactive Oxygen Species During Cryopreservation

The reactive oxygen species (ROS), especially superoxide anion (O_2^-), nitric oxide (NO), and hydrogen peroxide (H_2O_2), are involved in the events proceeding to capacitation (Belen et al. 2000). ROS can be generated by intracellular peroxidases and oxidases or by other enzymes like nitric oxidase synthase or cytochrome p450, though the chief source is the electron leakage from the electron transport chain (Ford 2004a, b). The plasma membrane redox system reduces oxygen and acts as the chief source of ROS generation in cells. NADH/NADPH oxidase could present in spermatozoa as it occurs on several other cell types (de Lamirande and O'Flaherty 2012). Especially, the death of ram and bull spermatozoa activates the aromatic amino acid oxidase that has been recognized as the main source of ROS generation (Chandra et al. 2012). This oxidase from dead spermatozoa in egg yolk extender impairs the motility and viability of the other viable bull spermatozoa. The contamination of leukocytes and spermatozoa preparation also acts as a huge source of ROS (Ford 2004a, b). Cooling to 4°C reportedly increased the ROS production by both

human spermatozoa and seminal leukocytes (Wang 2012). The disrupted equilibrium of ROS production and detoxification by antioxidants create oxidative stress. ROS like H_2O_2 arrest spermatozoa motility and hamper oxidative metabolism. In addition, ROS impedes the ability of spermatozoa to penetrate oocytes and block spermatozoa-egg fusion through the spermatozoa-SH groups' oxidation (Mammoto et al. 1996; Moawad et al. 2017). ROS can also cause spermatozoa DNA damage that has serious effects on post-fertilization events of early embryonic development (Lewis and Aitken 2005). Further, frozen-thawed spermatozoa are easily peroxidized than fresh spermatozoa in bulls (Tuncer et al. 2010). Spermatozoa membranes laden with unsaturated fatty acids are highly susceptible to this kind of peroxidation damage that results in a destabilized membrane.

12.3.6 Spermatozoa Chromatin Damage

Spermatozoa chromatin structure and DNA are vital for the paternal gene transfer to the oocyte and sustain the embryo development, but they are reported to be altered during cryopreservation (Ballachey et al. 1988; Hammadeh et al. 2001; Donnelly et al. 2001; Fraser and Strzezek 2004; Peris et al. 2004). The methods and stage of cryopreservation and the type of extenders used also influence chromatin damage. The cryopreservation can cause irregular chromatin condensation in human (Hammadeh et al. 2001; Donnelly et al. 2001), boar (Fraser and Strzezek 2004), and ram (Peris et al. 2004) spermatozoa. Freezing and thawing affect the DNA integrity and make them susceptible to epigenetic modification (Lewis and Aitken 2005). DNA fragmentation was reported to be occurring after freeze-thawing rather than equilibration (Elango et al. 2021). Cryopreservation-induced DNA fragmentation happens through excessive ROS production, diminished DNA repair enzyme functions, and stress on genomic regions of DNA.

12.3.7 Spermatozoa RNA and Protein Damage

Previously, it was believed that spermatozoa only transfer DNA to the oocyte, but later it was found that spermatozoa also carry mRNAs along with other small non-coding RNAs. The number of transcripts in spermatozoa significantly reduced after freeze-thaw (Valcarce et al. 2013). CIRBP transcript number was reduced, whereas the transcripts of CSPA (Cold shock protein A), heat shock protein 10 (HSP10), and HSP60 were increased after freeze-thaw (Wang 2012). The change in the quantity of transcript between fresh and frozen spermatozoa is due to the impact of freezing on mRNA-protein interaction that makes the mRNA more vulnerable to degradation (Valcarce et al. 2013).

   The microRNAs are small non-coding RNAs that are involved in various functions related to fertility. The differential expression of 55 microRNAs is also reported after freezing (Shangguan et al. 2020). CryomiRs (Cold-modulated miRNAs) are freeze-associated that can influence cold/freeze tolerance in mammals.

CryomiRs can accommodate the cells to new conditions and cause freeze tolerance by decreasing ATP consumption transcript expression and by regulating the reversible phosphorylation of metabolic enzymes (Biggar et al. 2009; Storey and Wu 2013). The mechanism of protein tyrosine phosphorylation is shown in Fig. 12.2.

The effect of the cryopreservation process on spermatozoa proteome was studied on boar (Pérez-Patiño et al. 2019), ram (He et al. 2019), rooster (Cheng et al. 2015), and human being (Bogle et al. 2017). They reported that the process of cryopreservation causes quantitative changes in the proteome of spermatozoa. Further, the cryopreservation process modifies the abundance of proteins related to membrane integrity, apoptosis, metabolism, motility, capacitation, and fertilization. Proteomic profiling of fresh, equilibrated, and frozen spermatozoa revealed that spermatozoa extending and equilibration are accountable for significant spermatozoa proteome alteration during cryopreservation, in which the majority of the proteins are membrane-bound (Westfalewicz et al. 2015).

12.3.8 Mitochondrial Damage

Mitochondria are the powerhouse of cells, including spermatozoa, located in the mid-piece, which are the major energy provider for the spermatozoa by ATP, which is important for spermatozoa to sustain motility, viability, and fertilizing ability. Nevertheless, the mitochondrion is one of the chief cryo-susceptible organelles during cryopreservation. The proportion of spermatozoa, in given frozen-thawed semen, with high mitochondrial membrane potential (MMP) has been reported to be related to fertility (Paoli et al. 2011; Moraes and Meyers 2018). When the spermatozoa MMP was evaluated during different stages of cryopreservation, it was found that MMP was significantly reduced after the equilibration stage of cryopreservation itself (O'connell et al. 2002; Shah et al. 2016; Elango et al. 2021). Not only MMP that are altered, but mitochondrial membrane fluidity also altered during cryopreservation that in turn induce mitochondrial ROS production that can adversely affect mitochondrial function and cause DNA fragmentation (Said et al. 2010).

12.3.9 Protein Tyrosine Phosphorylation in Spermatozoa During Cryopreservation

The mature spermatozoa are specialized cells and are inactive transcriptionally and translationally. Spermatozoa is reliant on post-translational modifications, especially tyrosine phosphorylation, for their post-ejaculatory functions such as hyperactivation, capacitation, and acrosome reaction, which are vital to fertilization (Urner and Sakkas 2003; Naz and Rajesh 2004). Leyton and Saling (1989) first established the presence of tyrosine phosphorylation in mouse spermatozoa. Since then, several workers have stated the protein tyrosine phosphorylation in
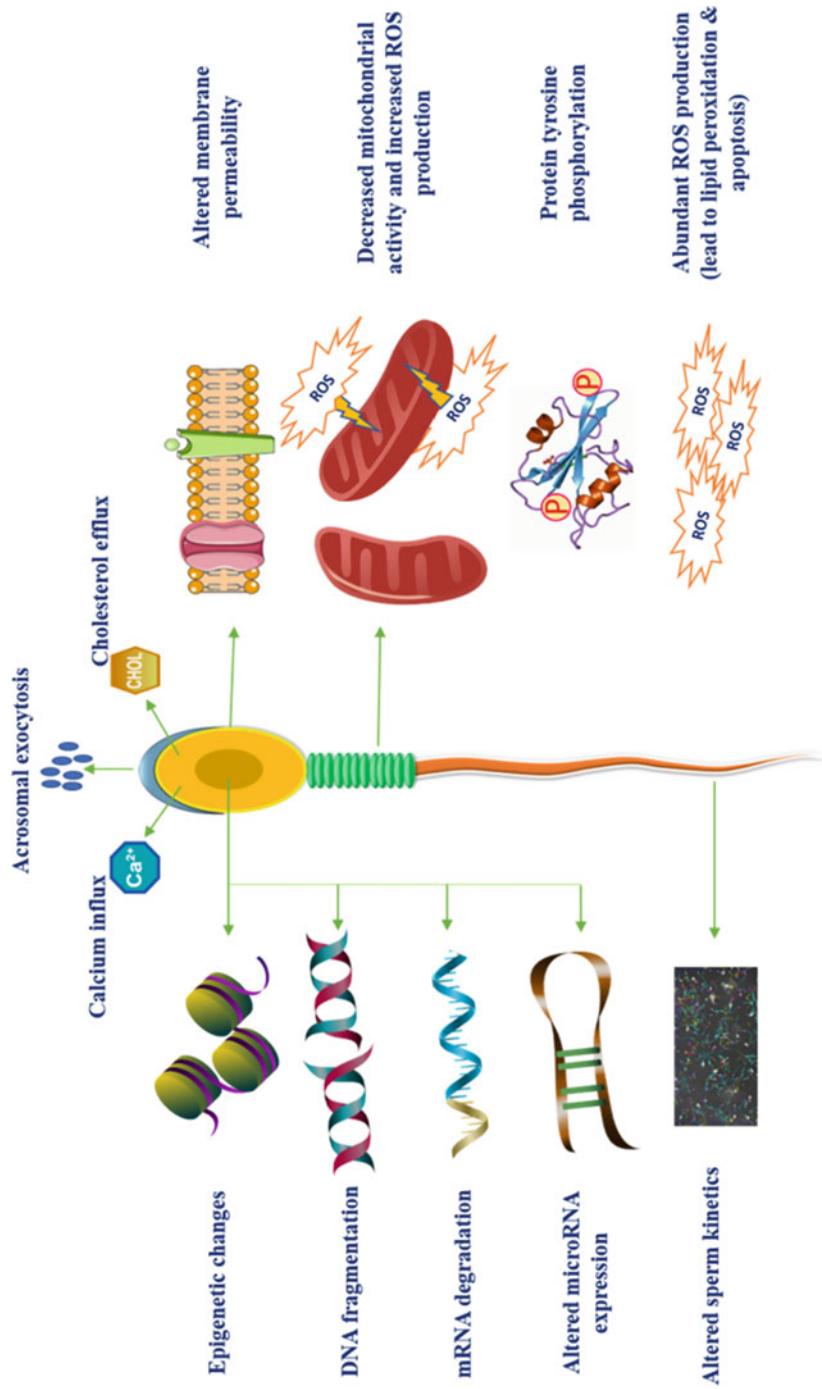
Fig. 12.2 Mechanism of protein tyrosine phosphorylation in spermatozoa

spermatozoa and its role in the fertilization process (Bailey et al. 2000; Kumaresan et al. 2012a, b).

In different species, including humans, mice, swine, and cattle, a clear relation between protein tyrosine phosphorylation and spermatozoa capacitation has been recognized. Intriguingly, such changes in protein tyrosine phosphorylation have also been observed in frozen-thawed boar (Tardif et al. 2001) and bull (Bailey et al. 2003) spermatozoa, indicating that cryopreservation can induce tyrosine phosphorylation of proteins. The majority of these studies established the phosphorylated proteins in spermatozoa by biochemical methods or by immune cytochemical evaluation of the different patterns of phosphorylated proteins. Not every spermatozoon undertakes tyrosine phosphorylation as a response to capacitation-inducing circumstances. Some of the subpopulations of spermatozoa display different vulnerabilities to tyrosine phosphorylation in humans, boar, and mice (Kumaresan et al. 2012a). Therefore, the quantification of the subpopulation of spermatozoa undertaking tyrosine phosphorylation during cryopreservation would be supportive in framing potent cryopreservation approaches, as studies exhibited that the tyrosine phosphorylation levels in post-thaw bull spermatozoa are related to their tolerance to cryopreservation and fertility (Kumaresan et al. 2012b).

12.4 How the Extenders Work?

The addition of a cryoprotectant into the semen is required to protect spermatozoa from cryodamage that happens during cryopreservation. Many varieties of extenders having various combinations of components (sugars, buffers, electrolytes, milk, milk products, and egg yolk) have been used for extending semen (Batellier et al. 2001). The extenders should be able to provide conducive pH (around 6.75–7.00), have buffering capacity, maintain suitable osmolarity, and essentially protect spermatozoa from cryo-injuries (Salamon and Maxwell 2000). Commonly, spermatozoa cryopreservation extenders include a non-permeating cryoprotectant (egg yolk, milk, lecithin), a penetrating cryoprotectant (glycerol, dimethyl sulfoxide, ethylene glycol), a buffer (Tris), one or more sugars (raffinose, glucose, lactose, saccharose, or trehalose), salts (sodium citrate, citric acid) and one or combination of antibiotics (penicillin, streptomycin, tylosin, lincomycin, spectinomycin, gentamycin,).

Based on the conformation and dynamics of the spermatozoa membranes, some substances like fatty acids, lipids, and proteins have been included in the extender with the aim of decreasing cryodamage to the spermatozoa. Egg yolk and glycerol extenders are among the first to be utilized for freezing semen (Holt 2000; Curry 2000; Medeiros et al. 2002), and nowadays also, many extenders have glycerol as the major cryoprotectant. The deleterious effects on spermatozoa during cryopreservation are due to changes in membrane fluidity, organization, and permeability, osmotic stress as well changes in the membrane lipid composition. Though glycerol is a cryoprotectant, it is toxic in higher concentrations and also in higher temperatures. Therefore, a balance needs to be done with regards to the length of time that the glycerol is in contact with the spermatozoa prior to freezing and the

concentration of glycerol to maximize the positive effects of glycerol as a cryoprotectant but reduce its toxic effects. The addition of 4% glycerol in an extender having 20% egg yolk was found to be good as compared to 2% or 6% glycerol in regard to progressive motility (Lemma 2011). Moreover, the effectiveness of glycerol may be affected by diluents and cryopreservation protocol.

The cryoprotectant nature of several other constituents, including liposomes and sugars, has also been established. Success has been stated with the use of trehalose as a cryoprotectant with a skimmed milk-egg yolk extender. Trehalose has the ability to maintain plasma membrane stability of spermatozoa (Ahmad and Aksoy 2012), thus having a synergistic effect when used with glycerol as a major cryoprotectant. Sucrose, raffinose, trehalose, lactose, and dextrans are impermeable to the plasma membrane of spermatozoa. Hence, it creates an osmotic pressure that can lower the incidence of intracellular ice formation, but induce cellular dehydration. The interaction of these sugars with phospholipids in the plasma membrane saves spermatozoa during cryopreservation (Aisen et al. 2002). Generally, monosaccharides have a greater cryoprotective effect than disaccharides when tried along with Tris (Purdy 2006).

The binding ability of egg yolk phospholipids to the spermatozoa membrane low density lipoproteins increases the permeability of the membrane, which reduces cryo-injury (Holt 2000). Liposomes have also been tried as a potential alternative cryoprotectant for bovine semen (Röpke et al. 2011).

Many extenders were established based on the above-mentioned principles, and they were used with varied success rates. They can be classified as follows:

1. Citrate-sugar-based extenders: Fructose, arabinose, and glucose were used with varied success rates. Occasionally, the citrate-sugar-based diluents were used for freezing semen from the late 1960s (Barbas and Mascarenhas 2009).
2. Lactose-based extenders: The use of lactose as the core component of diluents for freezing bull semen encouraged its application for the semen of other mammals. Lactose-yolk was used only for the non-glycerolated portion followed by glycerolated INRA medium or for both the glycerolated and non-glycerolized part of the diluents (Salamon and Maxwell 2000).
3. Milk extenders: Caseins are the protein fractions that are the basis of skimmed milk protection (Medeiros et al. 2002). Milk has been primarily used in reconstituted form combined with fructose, arabinose, or egg yolk for freezing mammalian semen, but adding egg yolk to heated homogenized milk did not help increase the post-thaw survival of spermatozoa (Salamon and Maxwell 2000).
4. Saccharose-based extenders: The saccharose has a better protective effect on acrosome integrity of spermatozoa than fructose, glucose, or lactose; therefore, it has been utilized as the chief component of many synthetic extenders (Sánchez et al. 2011).
5. Tris-based extenders: Tris extenders are frequently used for cryopreservation of semen of rams, bucks, and bulls. Ram spermatozoa can tolerate tris concentrations from 250 to 400 mM, and glucose is preferred over lactose, fructose, or raffinose as a sugar source (Salamon and Maxwell 2000). Tris with

375 mOsm/kg of osmolality and comprising 2% egg yolk was good in preserving motility and acrosomal integrity after thawing (Barbas and Mascarenhas 2009). The use of triladyl (Tris-based diluent) for trans-cervical insemination in sheep gave rise to good fertility. The in vitro results were better in this extender than saccharose-lactose-yolk and lactose-yolk. The addition of 2% bovine serum albumin improved the protective effect of triladyl on acrosome integrity.

6. Soybean-based extenders: The soybean extract-based extenders have been reported to have satisfactory cryoprotective capability during cryopreservation. Many studies have tried to cryopreserve spermatozoa with soy-based extender with varied success rates, and it needs more validation. Various commercial preparations like Bioexcell, Biciphos, Andromed, etc. comprise soy lecithin, and they are equivalent with the egg yolk-based extenders (Muiño et al. 2007; Arifiantini and Yusuf 2010; Kasimanickam et al. 2011; Akhter et al. 2011; Singh et al. 2012).

12.5   Antifreeze Proteins as Potential Cryoprotectant

Several organisms (ranging between bacteria, fungi, crustacean, microalgae, insects, and fish) thrive in sub-zero temperature due to the presence of a special kind of proteins, termed as antifreeze protein (AFPs) (Davies and Hew 1990; Gilbert et al. 2005; Krell et al. 2008; Raymond et al. 2009; Do et al. 2014; Robles et al. 2019). They can prevent ice recrystallization and bind with membrane, thus protecting the organisms from freezing injuries. This unique property, along with no toxic effect as glycerol, had made AFPs a potential candidate as a cryoprotectant for semen cryopreservation. Several groups try AFPs to cryopreserve semen from several species with varied success (Younis et al. 1988; Carpenter and Hansen 1992; Payne et al. 1994; Koshimoto and Mazur 2002; Prathalingam et al. 2006; Cabrita et al. 2014; Nishijima et al. 2014; Qadeer et al. 2014, 2015, 2016). AFPs increase osmotic resistance and prevent physical damage to the spermatozoa membrane. It binds to the spermatozoa membrane and prevents ice crystal formation by disallowing water molecules to join with each other. Apart from motility, the AFPs also improved different in vitro spermatozoa functions. However, due to the specific cytotoxic effects of the AFPs, the concentration in the extender needed to be optimized before large-scale use. Further variation between results observed in different studies has restricted large-scale adoption of the AFPs in cryopreservation.

12.6   Additives in Semen Extender

Different additives are tried with varied success in cryopreservation of bovine semen. Various additives tried in cryopreservation of bovine semen are recently reviewed extensively along with their mechanism of action (Debbarma et al. 2020). Different groups of additives used for bovine semen cryopreservation and their possible roles are enumerated in Table 12.1.

Table 12.1 Additives for semen cryopreservation and their roles

Group of additives	Role in preventing cryodamage
Antioxidants Glutathione (GSH), catalase, superoxide dismutase (SOD), carotenoids, vitamins C, vitamin E, vitamin B12, albumin, alpha-lipoic acid, EDTA, transferrin, melatonin	• protect spermatozoa from ROS-mediated lipid peroxidation of the spermatozoa membrane through either preventing formation of ROS or scavenging the already formed ROS
Antioxidant preservatives Butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), Tert-butyl hydroquinone	• act as ROS scavenger • prevent membrane and mitochondrial damages in the spermatozoa
Methylxanthines Pentoxifylline (PTX), caffeine	• stimulate the spermatozoa motility by inhibiting cyclic nucleotide phosphodiesterase (PDE) and preventing the inactivation cyclic adenosine monophosphate (cAMP) • neutralize ROS
Amino acids or proteins Glycine, cysteine, taurine, methionine, glutamine, proline, Cysteamine Buserelin acetate, Elamipretide, bovine serum albumin (BSA), Fetuin, Relaxin	• prevent oxidative damage during cryopreservation, thus protect spermatozoa plasma membrane during cryopreservation • few polypeptides affect different vital spermatozoa functions also
Cell membrane stabilizers Docosahexaenoic acid (DHA), alpha-linolenic acid (ALA), virgin coconut oil, cholesterol-loaded cyclodextrins	• provide stability and prevent damage to the membrane during cryopreservation
Trace elements Zinc, copper, manganese, selenium, iron, cobalt, iodine	• help in functioning of enzymes and proteins accompanying with fertility and male reproduction

Apart from the above, several natural ingredients such as Honey, extract of Pomegranate, Persimmon, Oats, Turmeric, Curcumin, Green tea, *Spirulina maxima*, Rosemary, Aloe vera, *Eurycoma longifolia*, etc. were also tested with varied success to prevent cryo-injuries.

12.7 Directional Freezing of Semen: a New Approach in Cryopreservation

Directional freezing has emerged as an alternative and efficient method for cryopreservation of semen (Arav and Saragusty 2016). The semen samples are frozen by passing through a temperature gradient at a particular speed to attain directional freezing. Samples in tubes or straws (in a broad volume range of 0.25 ml to 12 ml) are placed in the warm, highly insulated block and moved at a constant velocity from warm to cold gradient. This leads to highly controlled ice formation with very little cryodamage to the samples, largely dependent on the gradient of temperature and speed. As the sample moves through, the seeded ice crystals propagate in a highly controlled and regular pattern minimizing spermatozoa damage during freezing,

causing reduced mechanical damage and osmotic stress. This method enables the freezing of large volume semen. In cattle with a comparable conception rate with conventionally frozen semen doses, successful conception happened. The technology has successfully been tried in multiple wild and domestic species (Gacitua and Arav 2005; Si et al. 2006; Reid et al. 2009; Saragusty et al. 2009; Si et al. 2010). Nevertheless, the technology has to pass through several scanners of field fertility trials on a large scale to become commercially used. Though it has promised to be an efficient cryopreservation method, it is yet to be used commercially in the bovine industry.

12.8   Conclusions

Science is evolving faster than ever, and cryobiology is no exception. With the evolution of better equipment and modification of cryopreservation, bovine semen cryopreservation has become a routine process practiced in commercial-scale production. Even after decades of research in the cryopreservation of semen, still, a large proportion of spermatozoa become dead after the freeze-thaw cycle. This opens the avenue to search for an alternate cryopreservation method or alternate semen extender. Several commercial companies are developing defined extenders without animal protein, which may open a new avenue in this field. With the emergence of technologies, like directional freezing and antifreeze proteins which hold the potential to be a game-changer, the scenario may drastically change in the coming time.

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Buffalo Semen Cryopreservation: An Update

13

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Abstract

In spite of improvements in sperm cryopreservation techniques, conception rate using frozen-thawed sperm is not satisfactory. During the cryopreservation process, around 50% of sperm died and in the remaining 50%, motile sperm undergoes capacitation-like changes resulting from the poor fertilizing ability of buffalo spermatozoa; to improve the fertilizing ability of frozen-thawed sperm, a number of procedures, cryoprotectants, and additives are being used in the semen extender with different success rates. Recently, the use of egg yolk in the semen extender has raised many concerns like the presence of a high concentration of progesterone and microbial contamination in egg yolk. In the chapter, we summarized the factors which affect buffalo sperm during cryopreservation, attempted to improve buffalo sperm cryopreservation by different researchers, and finally discussed briefly the use of CASA for sperm evaluation and flow-cytometer for buffalo sperm sorting.

13.1 Introduction

The water buffalo is an important species within the Bovidae family. India harbors 109.85 million buffalo which is around 57% of the world's buffalo population (19th livestock census, Department of Animal Husbandry, Dairying and Fisheries, Govt.

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of India). India continues to be the largest milk producer in the world, of which around 52% is contributed by buffalo despite their less population as compared to cattle. The National Bank for Agriculture and Rural Development (NABARD) analyzed the impact of the use of frozen-thawed sperm for artificial insemination and found that the conception rate is increased from 20% to 35% only (2019). Poor semen quality is the major reason for the low conception rate. In buffalo, Roy and coworkers reported freezing of buffalo semen for the first time in 1956. Bagirov, in 1964, first reported pregnancy with frozen-thawed buffalo bull spermatozoa followed by several advancements in buffalo semen cryopreservation strategies occurred. In this chapter, the contributing factors affecting buffalo sperm cryopreservation, different additives, cryoprotectants, extenders, removal of seminal plasma, equilibration time, sperm encapsulation, sperm freezing drying, and use of sex-sorted cryopreserved sperm that is utilized to improve the freezability and fertility of buffalo spermatozoa have been discussed.

13.2   Research Update on Factors Affecting Buffalo Semen Cryopreservation

13.2.1 Estimation of Capacitation-like Changes, Cholesterol, Membrane Fluidity, and Intracellular Calcium in Fresh and Frozen-Thawed Buffalo Sperm

Sperm cryopreservation is a valuable tool for using elite buffalo bull for breed improvement but the fertilizing capabilities of buffalo sperm are greatly reduced, possibly due to premature capacitation during the process. Kadirvel et al. (2009) estimated premature capacitated sperm, damaged acrosome, membrane fluidity, and intracellular calcium in fresh and frozen-thawed semen. The results revealed that a significant percentage of buffalo spermatozoa were damaged during the cryopreservation process (Table 13.1). Normally, intracellular calcium in sperm increases in the female reproductive tract which triggers hyperactivation, capacitation, and acrosomal reaction to fertilize the ovum, but during cryopreservation these phenomena result in poor fertilizing ability of sperm.

13.2.2 Leakage of Enzymes from Buffalo Sperm During Storage of Cryopreserved Sperm

It is a general notion that cryopreserved sperm can be stored in liquid nitrogen for an indefinite time. Kar et al. (2015) estimated various antioxidant enzymes in frozen-thawed buffalo sperm and extended seminal plasma. They found that the superoxide dismutase (SOD) level decreased in the sperm and increased in the extended seminal plasma in a time-dependent manner. The catalase and glutathione peroxidase enzymes were not detected in frozen-thawed sperm, but detected in fresh sperm and extended seminal plasma. The study indicates that during the storage of

Table 13.1 Effect of sperm cryopreservation on capacitation-like changes, membrane fluidity, and intracellular calcium

	Fresh sperm	Frozen-thawed sperm
Capacitation-like change (%)		
Uncapacitated sperm	73.73 ± 2.73	36.75 ± 1.37
Capacitation-like change	14.32 ± 0.81	42.21 ± 2.23
Damaged acrosome	9.82 ± 0.64	23.34 ± 1.31
Sperm cholesterol ($\mu g/10^8$ sperm)	21.67 ± 1.36	12.89 ± 1.03
Sperm membrane fluidity (%)		
Live sperm with low fluidity	59.13 ± 2.47	17.85 ± 0.94
Live sperm with high fluidity	25.67 ± 1.23	53.62 ± 2.86
Dead sperm	17.43 ± 0.87	27.61 ± 2.45
Intracellular calcium (%)		
Live sperm with low calcium	55.23 ± 3.21	17.52 ± 1.23
Live sperm with high calcium	11.72 ± 0.54	43.68 ± 2.65
Dead sperm with low calcium	26.25 ± 1.67	19.12 ± 1.32
Dead sperm with high calcium	6.30 ± 0.34	20.32 ± 1.56

cryopreserved sperm, there are leakages of some enzymes into the extended seminal plasma. Further, the study put a question mark on the statement that the cryopreserved sperm can be stored in liquid nitrogen for an indefinite period. But, to reach a conclusion, there is a need for exhaustive studies in the area to reach a conclusion. Glutathione- S-transferases (GST) in sperm neutralize the free radicals generated during cryopreservation. Kumar et al. (2014) identified five and eight isozymic forms of GST and showed a significant amount of the enzymes are lost during the cryopreservation process.

13.2.3 Effect of Cryopreservation on Cytoskeleton Actin of Buffalo Sperm

The control of sperm cell volume during the cryopreservation process is essential to survive against cryo-stress. The sperm cell volume is controlled by cytoskeleton proteins mainly Actin and α-tubulin. Naresh and Atreja (2015) investigated the expression of actin in fresh, cooled, and frozen-thawed buffalo sperm and found that expression of actin was highest in fresh sperm and lowest in frozen-thawed sperm. The study indicates that actin polymerization is stimulated during the cooling as well as the cryopreservation process. Further, they found that the protein was localized in the flagellar and acrosome region. Thus, alteration in actin protein affects sperm motility and capacitation. The study is useful to improve the cryopreservation strategy to improve sperm fertilizing ability.

13.2.4 Cryogenic Changes in Proteases and Antiprotease Activities

At the time of fertilization, the sperm secretes acrosin and hyaluronidase that help in the digestion of the zona matrix that facilitates entry of the sperm through the zona pellucida. However, diluting semen with semen extender naturally presents proteases and protease inhibitors in the seminal plasma get diluted. The inhibitors are secreted by the accessory sex gland and mixed with the sperm at the time of ejaculation. However, how the enzymes act exactly is still unknown. The proteases and their regulators have been reported to involve in sperm maturation, storage, and activation. During cryopreservation, some enzymes like hyaluglucoronidase, glutamic oxalotransamidase (GOT), and glutamic pyruvate transamidase (GPT) are leaked from buffalo spermatozoa. In many studies, it is reported that there is a positive correlation between the leakage of hyaluglucoronidase, GOT, and GPT and the acrosomal damage. In buffalo, cryogenic changes in proteases and protease inhibitors have been characterized and found to be associated with sperm motility and fertility (Gurupriya et al., 2014). They showed two major proteases (45 and 42 kDas) and three minor proteases (95, 52, and 33 kDa) in buffalo spermatozoa. In seminal plasma, they found three major proteases (78, 68, and 62 kDa) and one minor protease (98 kDa) in buffalo seminal plasma. Interestingly, they disclosed that during cryopreservation, the activity of a major protease band of 52 kDa reduced progressively during the period of cryopreservation. In addition to it, during cryopreservation of buffalo spermatozoa, a new protease of 45 kDa leaked from buffalo spermatozoa to extended seminal plasma. In this way, they showed a number of proteases were identified in buffalo sperm and seminal plasma and demonstrated their activities during cryopreservation.

13.2.5 Antioxidant Enzymes in Fresh and Frozen Buffalo Sperm

Buffalo sperm are more prone to oxidative damage as compared to bull sperm because of their rich amount of polyunsaturated fatty acids. The main antioxidants present in spermatozoa are superoxide dismutases (SOD), catalases (CAT), and glutathione peroxidase (GSHPx). Kadirvel et al. (2014) found that SOD activity decreased by 47.7%, GSHPx by 62.7%, and GSH by 58.6% in frozen-thawed spermatozoa as compared to fresh spermatozoa (Table 13.2). Lone et al. (2018)

Table 13.2 Effect of cryopreservation on antioxidant enzymes and lipid peroxidation (Kadirvel et al. 2014)

Enzymes	Fresh sperm	Frozen sperm
SOD (U/10^9 sperm)	23.34 ± 11.78	11.78 ± 0.88
Catalase (U/10^9 sperm)	0.80 ± 0.01	0.00
GSHPx (U/10^9 sperm)	9477.32 ± 24.78	5276.43 ± 18.34
GSH (nmole/10^9 sperm)	23.46 ± 0.87	15.36 ± 0.43
LPO (nmol MDA/ 10^9 sperm)	278.78 ± 18.28	364.67 ± 22.40

Table 13.3 Sperm antioxidants and oxidants of buffalo semen (Lone et al. 2018)

	Fresh	Prefreeze	Frozen-thawed
SOD (units/mg protein)	0.52 ± 0.01	0.39 ± 0.06	0.16 ± 0.03
GPx (nmol min^{-1}ml^{-1})	91.60 ± 37.71	111.96 ± 39.85	59.82 ± 21.97
CAT (mM/mg protein)	0.31 ± 0.07	0.042 ± 0.00	0.001 ± 0.00
TAC (μM)	1.82 ± 0.13	1.71 ± 0.11	1.34 ± 0.09
LPO (nM MDA)	255.46 ± 15.83	297.87 ± 22.61	496.02 ± 39.28
ROS (units of H2O2)	81.80 ± 15.51	121.72 ± 11.58	197.02 ± 14.95

SOD superoxide dismutase, *GPx* glutathione peroxidase, *CAT* catalase, *TAC* total antioxidant capacity, *LPO* lipid peroxidation, *ROS* reactive oxygen species.

also reported SOD activity reduced at equilibration and post-thaw stages compared to fresh sperm (Table 13.3).

13.3 Attempts to Improve Efficiency of Buffalo Sperm Cryopreservation

13.3.1 Deoxygenation of Extender

The presence of abundant polyunsaturated fatty acids (PUFA) in the buffalo sperm plasma membrane is responsible for sperm damage. The binding of PUFA with sperm results in the production of reactive oxygen species. The environmental oxygen in the semen dilutor makes sperm susceptible to cold shock damage. Before freezing, buffalo sperm are exposed to aerobic conditions, but have a small number of antioxidants to counteract ROS in comparison to cattle sperm. The buffalo sperm are again exposed to oxygen and visible light radiation at the time of artificial insemination. When sperm are exposed to 95% oxygen than nitrogen, they lose sperm motility which rapidly suggests sperm suspensions produce ROS. During the preparation and handling of the semen extender, the dissolved oxygen is increased in the semen extender. Further, the filling, sealing, and printing of straws exposed sperm to environmental oxygen. The presence of excess oxygen has an adverse effect on sperm freezability. Balamurugan et al. (2018) conducted a study on partial deoxygenation of extender on buffalo sperm quality. They deoxygenated semen extender in two ways: partially deoxygenated by liquid nitrogen flushing and partially deoxygenated mechanically by a vacuum pump and found improvement in sperm motility and decline in lipid peroxidation and ROS production in freeze-thawed sperm compared to the control group.

13.3.2 Freeze-Drying of Buffalo Bull Spermatozoa

Sperm freeze-drying is an alternative technique of sperm cryopreservation. The freeze-drying process requires low cost, no liquid nitrogen, and little space for

storage. In different species, sperm freeze-drying has been documented and there are few reports for buffalo sperm. Shahba et al. (2016) attempted to freeze-dry the buffalo sperm. They cooled sperm cell suspensions in liquid nitrogen vapor for 1 h and plunged them in liquid nitrogen. The frozen samples were transferred in a programmable freeze-dryer stabilized at (-55 °C) and 0.001 mbar pressure. After thawing, freeze-dried sperm failed to preserve its motility.

13.3.3 Optimal Osmotic Pressure of Tris Buffer for Buffalo Semen Cryopreservation

Cattle sperm has an osmolality of 300 mOsm/kg, whereas buffalo bull sperm has an osmolality of 268 mOsm/kg (Khan and Ijaz, 2008). Because buffalo sperm has a lower osmolality than cattle sperm, cryopreservation of buffalo bull sperm in such extenders causes spermatozoa damage (Meyers, 2005). Semen extension in hypotonic or hypertonic solutions impacts spermatozoa by causing them to swell or shrink when the water moves across the plasma membrane. Higher motile spermatozoa were discovered in diluted semen samples with an osmotic pressure of 295 mOsm/kg, according to Mughal et al. (2013). They suggested that osmotic pressure of 285-295 mOsm/kg be used to account for the majority of spermatozoa features.

13.3.4 Use of Cholesterol-Loaded Cyclodextrin in Buffalo Cryopreservation

Cholesterol is a hydrophobic substance that is insoluble in aqueous diluents used to dilute sperm. To insert or remove cholesterol from synthetic and cell membranes, cyclodextrin has been employed. Cyclodextrins are cyclic oligosaccharides made from starch that have an exterior hydrophilic face and an internal hydrophobic core, allowing them to encapsulate hydrophobic substances like cholesterol. Rajoriya et al. (2014) studied the effect of cholesterol-loaded cyclodextrin (CLC) on buffalo sperm freezability and discovered that CLC treatment preserves the C/P ratio and is critical for spermatozoa membrane architecture (Table 13.4).

Table 13.4 Cholesterol, phospholipids, and its ration in buffalo sperm (Rajoriya et al. 2014)

	Cholesterol ($\mu g/100 \times 10^6$ sperm)		Phospholipids ($\mu g/100 \times 10^6$ sperm)		Cholesterol/ Phospholipids	
	Prefreeze	Post-thaw	Prefreeze	Post-thaw	Prefreeze	Post-thaw
Fresh sperm	21.55	13.18	90.48	79.45	0.24	0.62
CLC (3.0 mg/120 $\times 10^6$ sperm)	55.67	36.21	90.23	82.35	0.17	0.44

Abouelezz et al. (2017) investigated sperm DNA damage and ultrastructure in buffalo bull spermatozoa vitrified with cholesterol-loaded cyclodextrins in the presence or absence of cholesterol (CLC). These results imply that cholesterol loss is a plausible cause of poor buffalo semen quality after cryopreservation, and they show that altering lipid content during cryopreservation is a promising technique for improving buffalo semen quality.

13.3.5 Equilibration Time for Buffalo Sperm

Equilibrium time refers to the total time that sperm is exposed to glycerol before freezing. At this stage, glycerol enters the sperm to establish an equilibrium concentration inside and outside the cell. It should be remembered that the equilibration process applies not only to glycerol, but also to other osmotically active ingredients. The equilibration can therefore be influenced by the type of elongation agent (buffer and cryoprotectant) and the subsequent freezing procedures. Shahverdi et al. (2014) demonstrated that a longer equilibration time of 2 h resulted in better preservation of buffalo sperm parameters during cryopreservation. However, there was no significant interaction between the 4 h equilibration time and the type of extender (yolk and Bioxcell), resulting in higher sperm viability after thawing. Tuli et al. (1981) reported that the viability of buffalo spermatozoa in Tris egg yolk glycerol extender was better at all stages using 4 h freezing compared with 0 and 2 h equilibration freezing times.. However, equilibrating 2 h at 5 °C compared with 0 h improved thaw recovery, incubation survival, and reproductive rates of frozen buffalo semen (Dhami et al. 1996). It should be mentioned that buffalo sperm can be stored at 5 °C for at least 24 h without significant reduction in motility and fertility (Sansone et al. 2000). The thawing quality of buffalo epididymis was also unaffected by the change in equilibration time (range: 29 h) following soy lecithin prolongation. Most cryopreservation protocols for buffalo sperm suggest an equilibration period of 4 h, thus the semen has to be frozen on the same day of collection.

13.3.6 Alginate Encapsulation

The use of artificial insemination (AI) in buffalo is limited by low ovarian activity in the hot season, qualitative seasonality in semen, low sperm resistance in the main tract, and difficulty in estrus detection and estrous timing change. Coating sperm with alginate can limit sperm damage and prolong sperm release time. However, this technology has not yet reached field conditions because encapsulated sperm are not commercially available. For the first time, the sperm encapsulation technique was applied by Perteghella et al. (2017) in buffalo and there is no standard method for cryopreservation of encapsulated buffalo sperm. Perteghella et al. (2017) found that the percentage of motile bovine spermatozoa was reduced by encapsulation, but pregnancy rates for encapsulated sperm were equal compared with those in the

control group. These results represent an encouraging starting point for the development of other strategies, thus meeting the needs of buffalo breeding programs.

13.3.7 Removal of Seminal Plasma

Albuquerque et al. (2017) evaluated the semen quality of buffalo by two methods of sperm removal (filtration and centrifugation) and found that plasma removal did not improve sperm kinetics. Centrifugation increased the number of cells with damaged acrosomal membranes, and filtration caused plasmalemma damage and larger acrosomal membranes. In contrast, ROS production was higher in the centrifugation group than in the control and filtration groups, although no difference in TBARS formation was detected. Therefore, sperm removal did not improve the quality of thawed buffalo spermatozoa compared with controls in terms of sperm kinetics, membrane integrity, mitochondrial membrane potential, or lipid peroxidation. The hypothesis that plasma removal causes less sperm damage and improves buffalo semen quality remains unconfirmed and further studies are needed in this regard.

13.4 Use of Different Permeable Cryoprotectants for Cryopreservation of Buffalo Sperm

13.4.1 Dimethyl Sulfoxide (DMSO)

Despite the toxic effects of glycerol, it is a popular cryoprotectant for the cryopreservation of buffalo sperm. DMSO has the ability to penetrate cells faster than glycerol. Rasul et al. (2007) investigated the synergistic effect of DMSO and glycerol when added at different temperatures on post-thawing buffalo sperm quality. The addition of glycerol (6%) at 37 °C gave better sperm motility after thawing, better plasma membrane integrity, and better velocity than addition at 4 °C. It was concluded that the addition of DMSO antagonized the cryoprotective capacity of glycerol and reduced the quality of buffalo spermatozoa after thawing. In addition, 6% glycerol added at 37 °C provided better cryoprotection for the motile apparatus and plasma membrane integrity of buffalo spermatozoa. The interaction of glycerol and DMSO showed that in the absence of glycerol, DMSO did not protect sperm motility or significantly protect sperm plasma membrane during cryopreservation. Even at lower concentrations, DMSO (1.5%) antagonized the cryoprotective effects of glycerol, reducing post-thawing motility and plasma membrane integrity of buffalo spermatozoa. The antagonistic activity of DMSO on thawed sperm motility and plasma membrane integrity worsened with an increasing concentration of glycerol in the extender. Therefore, DMSO in combination with glycerol in Tris-based extender had a deleterious effect on the post-thaw motility and plasma membrane integrity of buffalo sperm. Glycerol (6%, v:v), when added at 37 °C, provided a better cryoprotection to sperm motility and membrane integrity of buffalo sperm. Therefore, glycerol is a better cryoprotectant of choice for freezing buffalo sperm.

Table 13.5 Use of permeable cryoprotectants other than glycerol in cryopreservation of buffalo sperm

Cryoprotectants	Dose	Results	References
Ethylene glycol	5%	Better than glycerol	Swelum et al. (2011)
	5%	Not better than glycerol	Rohilla et al. (2005)
	5,7 and 10%	Not better than glycerol	Abdel-Khalek et al. (2008)
	3, 5 and 7%	Did not differ	Tuli et al. (2004)
DMSO	1.5%	Poor than glycerol	Rasul et al. (2007)

13.4.2 Ethylene Glycol

The development of less toxic cryoprotectants could significantly contribute to improving the quality of frozen and thawed buffalo semen. Ethylene glycol can be used to freeze bubaline sperm as an alternative to glycerol. But other studies (Tuli et al., 2004; Rohilla et al., 2005) found no improvement after using ethylene glycol for cryopreservation of buffalo semen (Table 13.5).

13.5   Use of Additives to Improve Post-Thaw Semen Quality

13.5.1 Cysteine

Cysteine and glutamine are major components of glutathione, which have an antioxidant role in blocking the lipid peroxidation activity of ROS in sperm. Cysteine is an amino acid and a precursor molecule of glutathione, which is an intracellular antioxidant that protects cells from the harmful effects of ROS on cell organelles. On the other hand, cysteine is composed of thiol groups, which act individually as nonenzymatic antioxidants and readily penetrate the semen. Semen freezing significantly reduces the glutathione content of bull semen. Beneficial effects of cysteine (Ansari, 2011; Beheshti et al., 2011) and glutamine (Elkhawagah et al. 2015) have been reported in cryopreservation of buffalo semen. Topraggaleh et al. (2014) found that cysteine at a concentration of 7.5 mmol increased progressive motility and mitochondrial membrane potential, compared with control in buffalo sperm. Furthermore, they showed that the addition of 7.5 mmol cysteine and 15 mmol glutamine in sperm cryopreservation extender was more likely to decrease intracellular ROS and subsequently increase motility and membrane integrity of frozen and thawed buffalo sperm.

13.5.2 Curcumin (Diferuoyl Methane)

Curcumin (diferuoyl methane), a natural radical scavenging antioxidant, is a yellow compound isolated from the dried rhizome of the perennial herb Curcuma longa L. of the family Zingiberaceae. From cyclic charge studies, Hatom transfer from the

CH2 group to the center of the heptadione bond has been shown to have an antioxidant role in curcumin as well as a role for its phenol–OH group. Conversion of the 1,3 dicarbonyl radical of curcumin to an isosteric heterocycle as in pyrazole curcumin reduces its rotational freedom, leading to enhanced redox properties as well as its antioxidant activity. Shah et al. (2017a, b) found that the coagulation capacity of water buffalo sperm was enhanced by the addition of 1.5 mM curcumin in the semen dilutor.

13.5.3 Taurine and Trehalose

The sulfonic amino acid, taurine, acts as an antioxidant and can cross the plasma membrane of sperm and inhibit lipid peroxidation while protecting cells against ROS accumulation. Trehalose, a non-reducing disaccharide, has a protective role against osmotic effects and forms specific interactions with membrane phospholipids, making the medium hypertonic, causing dehydration to the cell before solidification, and thus reducing the number of cells injured by ice crystallization. Reddy et al. (2010) demonstrated the cryoprotective effects of taurine and trehalose on buffalo sperm quality parameters after freezing-thawing. Buffalo semen was cryopreserved in Tris-based egg yolk diluent with cryoprotectants such as taurine (50 mM) or trehalose (100 mM) and used to evaluate sperm quality parameters such as motility, viability, plasma membrane integrity, total antioxidant status, and degree of cryo-preservation. Evaluation of spermatozoa after thawing indicated that the addition of taurine or trehalose significantly improved motility, viability, and membrane integrity and that the degree of frozen sperm was significantly reduced in the presence of taurine or trehalose.

13.5.4 Regucalcin

Regucalcin (RGN) affects Ca 2+ homeostasis by controlling the activity of the Ca 2+ pump in the cell membrane, nucleus, microsomes, endoplasmic reticulum, and mitochondria. RGN is widely distributed in the male reproductive tract of buffalo (Pillai et al., 2017a, b c) and is found in the seminal fluid of buffalo (Pillai et al. 2017b). A concentration of 40 µg/ml of recombinant RGN added during sperm freezing increased sperm motility after progressive thawing, acrosome integrity, and zona pellucida-associated spermatozoa. Thus, approximately 1 µM of recombinant RGN, which retains calcium-binding capacity, exerts a cryoprotective effect on buffalo spermatozoa as an extender.

13.5.5 Arachidic Acid

In buffalo semen, cold shock and freezing significantly resulted in a significant loss of total lipids (15.8% and 34.55%) and phospholipids (6.49% and 19.1%),

respectively (Sarmah et al. 1984). The major loss of fatty acids from the sperm membrane may be due to the production of acetyl CoA through beta-oxidation and lipid peroxidation reactions. The ratio of unsaturated fatty acids to saturated fatty acids determines the susceptibility of sperm to cold shock, and species with such a high ratio are more sensitive to cold shock. Buffalo semen has a higher plasma membrane polyunsaturated fatty acid content than bull semen, making them more susceptible to cold shock and freezing/thawing injury. The higher content of poly-unsaturated fatty acids in the plasma membrane is thought to confer higher fluidity and low resistance to cold shock due to the presence of multiple double bonds making them susceptible to peroxidation. In contrast, saturated fatty acids are less susceptible to peroxidation than unsaturated fatty acids. The incorporation of saturated fatty acids into plasma membrane phospholipids may increase saturation and reduce the risk of lipid peroxidation, which may compensate for damage caused by freezing. An arachidic acid is a saturated form of arachidonic acid that occurs naturally in buffalo semen. Ejaz et al. (2016) hypothesized that the addition of a saturated fatty acid, arachidic acid, could improve sperm quality after thawing of sperm of Nili Ravi bulls. Ejaz et al. (2016) evaluated the effect of arachidic acid supplementation at different concentrations in buffalo semen. The results showed that progressive sperm motility (%) was improved in a dose-dependent manner by adding arachidic acid at 5.0, 10.0, and 20.0 ng/ml. Among the arachidic acid concentrations studied, the maximum improvement in semen quality parameters after thawing was observed at 20.0 ng/ml.

13.5.6 Relaxin

Relaxin, an insulin superfamily regulatory peptide, is an important plasma component negatively affected by cryopreservation (Lessing et al. 1985). Relaxin, produced by male accessory glands, is secreted into seminal plasma and saturates with sperm shortly after ejaculation. Elkhawagah et al. (2015) evaluated the effect of relaxin on the reproductive indices of frozen/thawed buffalo spermatozoa. Sperm were incubated without a capacitating agent and with known capacitating agents such as heparin and relaxin (50 and 100 ng/ml) for 2 and 4 h. Although viability was not affected, relaxin increased sperm motility compared with negative and positive controls. In addition, relaxin has beneficial effects on the sperm motility, capacitation, and fertility of frozen-thawed buffalo spermatozoa.

13.5.7 Royal Jelly Supplementation

Royal jelly (RJ) is secreted by the hypopharyngeal glands of worker bees mainly to feed the larvae and maintain the queen bee. It contains proteins, lipids, sugars, vitamins (A, B5, C, D, E), and essential amino acids, including cystine, lysine, and arginine. The amino acids present in RJ have been noted for their high antioxidant capacity and resistance to free radicals such as superoxide anion radicals.

Shahzad et al. (2016) evaluated the effect of royal jelly (RJ) on sperm quality after thawing, in vitro, and in vivo fertility rate of cryopreserved male buffalo spermatozoa. The results indicated that progressive sperm motility was significantly higher in the RJ-supplemented groups (0.05, 0.1, 0.2, and 0.3%) than in the control groups. Sperm viability, plasma membrane, and acrosome integrity were significantly improved with 0.1% RJ added; however, pregnancy rates were similar between the 0.1% RJ supplement group and the control group.

13.5.8 Sericin

Sericin is rich in aspartic acid as well as serine, which has a high content of hydroxyl groups. Studies have shown that sericin blocks lipid peroxidation, prevents cell death, and resists freezing and other types of stressors. Kumar et al. (2015a, b) examined the protective role of sericin on bovine spermatozoa during cryopreservation. Extender supplemented with 0.25, 0.5, and 1% sericin resulted in higher sperm motility and GPx activity. In addition, plasma membrane integrity and SOD activity were higher in the groups supplemented with 0.25 and 0.5% sericin. MDA concentrations were significantly lower in the 0.25 and 0.5% sericin-treated groups compared with the control and other treated groups.

13.5.9 Antifreeze Proteins

Antifreeze glycoproteins (AFGP) and antifreeze proteins (AFP) present in the blood and tissues of poikilotherm organisms inhibit the growth of ice crystals in a non-colligative manner. Antifreeze protein (AFP), a polypeptide, produced by polar fish, some insects, and plants, inhibits the formation of ice crystals by a non-colligative mechanism. Antifreeze proteins (AFPs) were first extracted from the serum of polar fish about four decades ago and were initially recognized as macromolecules that specifically attach to ice crystals and inhibit their growth. Based on the difference in amino acid sequence and tertiary structure, AFP has been classified into AFP III and antifreeze glycoprotein. AFP lowers the temperature at which the ice crystals increase in size in a non-colligative manner and thus exhibit thermal hysteresis. With regard to sperm preservation, studies have reported different effects of AFP in the diluent on cooled or cryopreserved sperm in different species. These effects ranged from adverse in rats to beneficial in chimpanzees, sheep, fish, cattle, and buffalo semen. Qadeer et al. (2014), supplementing with semen extender with AFP III at 0.1 μg/mL improves progressive sperm motility of buffalo semen. The increase in sperm motility with AFP addition suggests a possible effect by blocking ion channels and stabilizing the transmembrane electrolyte gradient, reducing calcium flux and stabilizing the membrane. Then, Qadeer et al. (2015) evaluated the effect of AFGPs1–5 and AFGPs 7–8 in semen extender on bovine spermatozoa after cooling and cryopreservation found that supplementation of 1 μg/ml of AFGP in extender improved the motility and plasma membrane

integrity of buffalo sperm. Finally, Qadeer et al. (2016) evaluated recombinant antifreeze protein (DAFP) from beetle (Dendroides Canadensis) for cryopreservation of buffalo sperm. The presence of antifreeze proteins is an important overwintering adaptation of many insects. In the freeze avoiding overwintering D. Canadensis larvae, these proteins are present in the hemolymph, gut, primary urine, and epidermal cells where they function to inhibit inoculative freezing and promote supercooling by inhibiting ice nucleators. The supplementation of 10 µg/ml of DAFP in the extender improved the motility and plasma membrane integrity of buffalo sperm after freeze-thawing and yielded numerically higher, although statistically nonsignificant, in vitro cleavage and in vivo fertility rate.

13.5.10 Resveratrol

Resveratrol is mainly found in grapes showing antioxidant, anticancer, antiaging, anti-inflammatory, cardioprotective, and neuroprotective actions. Longobardi et al. (2017a, b) evaluated supplementation of resveratrol in semen extender and found that inclusion of 50 µM resveratrol decreases capacitation-like changes and oxidative stress and improves in vitro fertilizing ability of buffalo semen.

13.5.11 Carnitine

Carnitine is synthesized in kidneys and liver from lysine and methionine. It is a strong antioxidant. Longobardi et al. (2017a, b) evaluated the effect of carnitine supplementation in buffalo semen extenders. The supplementation of carnitine (7.5 mM) in the semen extender significantly improved sperm motility and membrane integrity, reducing capacitation-like changes of buffalo sperm.

13.5.12 Insulin-Like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) is secreted by Leydig and Sertoli cells involved in spermatogenesis and steroidogenesis. Kumar et al. (2019a, b) studied the effect of IGF-1 supplementation on buffalo semen cryopreservation. Supplementation of IGF-1 (250 ng/ml) improved sperm motility, longevity, and membrane intactness after cryopreservation of semen.

13.5.13 Iodixanol

Iodixanol is a medium for density-gradient centrifugation. Saragusty et al. (2009) found that iodixanol protects sperm membranes and preserves sperm. Swami et al. (2017) determined the effect of iodixanol on buffalo spermatozoa and found that

iodixanol has the ability to protect spermatozoa during cryopreservation of buffalo sperm.

13.5.14 Mifepristone (RU 486)

The progesterone in egg yolk-based extender may be involved in triggering signaling pathways leading to capacitation-like changes. Dalal et al. (2019) showed that RU 486 (mifepristone) in semen extenders protects sperm to undergo capacitation like change during cryopreservation. They summarized that RU 486 prevents activation of CatSper channel and calcium influx, minimizes tyrosine phosphorylation, and prevents the initiation of capacitation during the cryopreservation process.

13.5.15 Sodium Alginate

Sodium alginates synthesized from brown seaweed are biocompatible, non-immunogenic, and nontoxic polymers. Kumar et al. (2019a, b) determined the effects of sodium alginate on buffalo sperm during cryopreservation. Supplementation of sodium alginate (0.4%) in extender improved sperm longevity and plasma membrane integrity and phosphorylation of tyrosine-containing proteins during cryopreservation. Also, malondialdehyde concentration of sperm was less in sodium alginate-treated sperm as compared with control samples. Supplementation with sodium alginate also improved the metal-chelating capacity and antibacterial properties of the extender. The details of various semen extender additives tested on buffalo semen are given in Table 13.6.

13.6 Different Sources of Egg Yolk for Cryopreservation of Buffalo Bull

13.6.1 Quail and Turkey Egg Yolk for Cryopreservation of Buffalo Bull

In comparison to chicken egg yolk, quail egg yolk has a great concentration of phosphatidylcholine, less phosphatidylethanolamine, and a lower ratio of polyunsaturated fatty acids to saturated fatty acids. The saturated fatty acids are less susceptible to lipid peroxidation than unsaturated fatty acids. Turkey egg yolk has a higher concentration of cholesterol compared to chicken egg yolk. Akhter et al. (2017) found that quail egg yolk at 5% and turkey egg yolk at 10% in semen extender proposition compensations over 20% chicken egg yolk in terms of buffalo sperm motility and fertility.

Table 13.6 Use of various additives in the cryopreservation of buffalo sperm

Additives	Dose	References
Cysteine	1 mm (L-cysteine)	Ansari et al. (2011)
	7.5 mmol cysteine	Topraggaleh et al. (2014)
Glutamine	15 mmol	Topraggaleh et al. (2014)
Curcumin	1.5 mM	Shah et al. (2017a, b)
Taurine	50 mM	Reddy et al. (2010) Singh et al. (2015)
Trehalose	100 mM	Reddy et al. (2010) Singh et al. (2015)
Relaxin		Elkhawagah et al. (2015)
Royal Jelly	0.1%	Shahzad et al. (2016)
Antifreeze protein III	0.1 µg/mL	Qadeer et al. (2014)
Antifreeze glycoproteins	1 µg/ml	Qadeer et al. (2015)
Resveratrol	50 µM	Longobardi et al. (2017a, b)
Carnitine	7.5 mM	Longobardi et al. (2017a, b)
Sericin	0.25 and 0.5%	Kumar et al. (2015a, b)
Idoxinol	2.5%	Swami et al. (2017)
IGF-1	250 ng/ml	Kumar et al. (2019a, b)
Mifepristone	10 µM	Dalal et al., 2019
Sodium alginate	0.4%	Kumar et al. (2019a, b)

13.6.2 Duck Egg Yolk for Cryopreservation of Buffalo Sperm

Artificial insemination using frozen-thawed semen has not shown good fertility results in buffalo (Kumaresan et al., 2005). Andrabi et al. (2008) compared duck egg yolk (DEY), Guinea fowl egg yolk (GFEY), and Indian indigenous hen (Desi) egg yolk (IDEY) in the semen extender for improving the post-thaw quality of buffalo bull spermatozoa and compared it with commercial hen egg yolk (CHEY). Among them, DEY was found to be a superior extender in terms of improvement of the quality of cryopreserved buffalo semen. Next, Waheed et al. (2012) compared chicken egg yolk (CEY) and duck egg yolk (DEY) in extenders and recommended that DEY may be replaced with CEY with the improvement of semen quality of cryopreserved semen in Nili-Ravi buffalo.

13.7 Use of Computer-Assisted Sperm Analysis (CASA) Parameters to Evaluate Semen Quality and Fertility of Cryopreserved Buffalo Sperm

Rasul et al. (2001) demonstrated that during the dilution and cooling steps of the cryopreservation process, straight-line velocity (VSL) and average path velocity (VAP) of buffalo spermatozoa remained unaffected but reduced significantly after the freeze-thaw step. Further, they demonstrated that lateral head displacement (ALH) of spermatozoa decreased during cooling, somewhat increase in the

Table 13.7 Mean of buffalo sperm motility, kinetic parameters, and viability of fertile and subfertile bulls (Kumar et al., 2014)

Parameters	Fertile bulls	Sub-fertile bulls
Total motility (%)	56.88 ± 3.2^a	43.82 ± 1.7^b
Progressive motility (%)	16.59 ± 1.1	16.57 ± 2.1
Rapid motility (%)	20.25 ± 2.5	20.35 ± 2.8
Slow motility (%)	30.62 ± 2.6^a	18.25 ± 1.4^b
VAP (μm/s)	87.22 ± 2.4^a	79.02 ± 1.6^b
VSL(μm/s)	68.93 ± 1.9^a	63.42 ± 1.2^b
VCL(μm/s)	156.52 ± 4.3^a	142.37 ± 2.8^b
ALH ((μm)	6.80 ± 0.1	6.57 ± 0.07
BCF (Hz)	33.98 ± 0.4	33.43 ± 0.5
STR (%)	79.21 ± 0.7	78.74 ± 0.6
LIN (%)	45.59 ± 0.4	45.50 ± 0.7

equilibration stage, and again reduced after the cryopreservation process. The linearity and straightness of spermatozoa increased after the cryopreservation process. The cryo-capacitation increases circular motility and lateral head displacement of spermatozoa.

13.7.1 CASA Parameters of Frozen-Thawed Fertile and Subfertile Buffalo Bulls

The fertility valuation of cryopreserved semen is required for the genetic improvement of buffalo through artificial insemination. The laboratory evaluation of semen from buffalo bulls is largely based on subjective scoring of sperm motility. Hence, an accurate, in vitro method is required to predict the fertility of buffalo. Kumar et al. (2014) found that the mean values of total motility, VAP, VSL, and VCL of sperm for fertile bulls were significantly higher than subfertile bulls (Table 13.7).

13.8   Flow-Cytometric Sorting of Buffalo Sperm

The sorting of spermatozoa for the desired sex of offspring is one of the important new biotechnologies tools available for livestock husbandry. For the first time, Presicce et al. (2005) sorted buffalo X and Y sperm by flow-cytometric method and cryopreserved. They inseminated the sexed spermatozoa and found equivalent conception rates to those of conventional AI. Lu et al. (2006) estimated the difference in DNA content characterizing the X- and Y-chromosome-bearing sperm of buffalo. The difference in DNA content between X- and Y-sperm is 3.59 and 3.55 on the basis of fluorescence intensity in Murrah and Nili-Ravi buffaloes, respectively. Lu et al. (2007) revealed that the accuracy of sorted X- and Y-buffalo sperm was 94% and 89%, respectively.

13.9 Concluding Remarks and Future Directions

While reviewing information on buffalo cryopreservation, there is a completely lacking information related to proteomics, transcriptomics, and epigenomics of cryopreserved buffalo sperm. The technologies may provide new insights into the mechanisms underlying the poor freezability and fertility of buffalo sperm. The technologies would also help in the detection of potential biomarkers for sperm freezability as well as for the development of new strategies to increase the efficiency of buffalo sperm cryopreservation. The transcriptomics study is required to increase our knowledge about how buffalo sperm cryopreservation affects different RNA transcripts involved in fertilization and embryo development. The supplementation in semen extender with new cryoprotectants, antioxidants, and other new components also requires further optimization of semen extender for buffalo sperm cryopreservation.

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Advances in Bovine Sperm Quality Assessment: From Motility to Fertility

14

K. Elango, S. S. Layek, and A. Kumaresan

Abstract

The success of artificial insemination and optimal use of genetically superior bulls is determined by the fertility of the bull, which in turn depends on sperm quality in frozen semen. Therefore, precise estimation of sperm fertilizing potential is important for achieving greater fertility in dairy bovines. There were the days when semen quality was assessed only by macroscopic assessments (Volume, color, consistency, density, pH), but then slowly progressed towards the microscopical assessment such as motility, morphology, and viability. Thereafter, sperm were analyzed under different types of microscopes such as light microscope, phase contrast microscope, differential inference contrast, transmission electron microscope, and cell phone microscope. Irrespective of the type of assessment used, results are invariably affected by subjectivity and less repeatability due to evaluator's skills. Therefore, conventional methods of semen analysis have limited value for fertility prediction. In the era of computerization and automation, many new methods are in place for sperm quality assessment with high sensitivity and specificity. The major breakthrough in semen analysis is development of fluorochromes that could help in assessment of subtle features of spermatozoa. With the advent of high-throughput analytical techniques like Flow cytometry, now, it has become possible to analyze several thousands of spermatozoa within short time. Due to these advancements and evolution in semen analysis, nowadays, plethora of tests is available for the evaluation of

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sperm quality that can help in precise prediction of sperm fertilizing potential, which are detailed in this chapter.

14.1 Introduction

"A" for "Antonie van Leeuwenhoek", when it comes to sperm evaluation, as he was the first to observe spermatozoa under microscope (Leeuwenhoek 1678). However, the need for sperm quality assessment was perceived at a later stage when the contribution of males towards infertility was realized; thanks to Mantegazza, who first correlated semen characteristics with fertility (Mantegazza 1866). Fertility of male animals is commonly assessed after mating or insemination of several females based on their ability to produce their progeny. Even though this is trustworthy, it is time-consuming and costly. Moreover, only lesser number of males can be tested by this method (Gillan et al. 2008). Therefore, dependence on semen evaluation techniques has become obligatory and development of techniques for the prediction of male fertility has become essential (Rodríguez-Martínez 2003). In between the days when spermatozoa were considered as seminal parasites and when it has become possible to assess multiple parameters of single spermatozoon, journey of semen evaluation inculcated lot many seminal parameters, techniques, and equipment and achieved varying degree of success in predicting the fertility.

In bovines, breeding bulls are generally selected for use in artificial breeding based on breeding soundness evaluation (BSE), but 20–25% difference in conception rates was reported among those selected bulls (Aslam et al. 2014) and significant proportion of bulls that have passed these tests produced poor quality ejaculates that are unfit for cryopreservation and use in artificial breeding (Thippeswamy et al. 2014). Traditional semen quality assessment in BSE includes motility, sperm morphology, and concentration of spermatozoa (Gillan et al. 2008). However, these tests are solely focusing on structural aspects of spermatozoa that are having minimal correlation with fertility (Rodríguez-Martínez 2013). Even though routine semen analysis and BSE provide useful information about sperm production, sperm motility, viability, patency of the genital tract, and secretions of the accessory organs, it remains as an imperfect tool in predicting male infertility, because it does not provide much information about the functional attributes of the spermatozoon to fertilize an ovum or to undergo the successive maturation processes required to attain fertilization (Vasan 2011; Vijetha et al. 2014). Though in vitro function tests such as cervical mucus penetration test, zona free hamster egg penetration test, hemi zona assay, and sperm nuclear chromatin decondensation test are capable of predicting functional potency of sperm to some extent, they evaluate only small quantity of

Table 14.1 Essential sperm attributes required to fertilize an oocyte

| Sperm attributes	Importance of attributes
Plasma membrane integrity	Vital for cell-to-cell interaction (sperm with oviductal epithelium or sperm with oocyte)
Acrosomal integrity	Intact acrosome is essential for the timely exocytosis of acrosomal contents during sperm-oocyte interaction
Chromatin integrity	Crucial for the fertilizing sperm to initiate and endure fertilization process; to substantiate embryonic development; important for offspring fitness
Mitochondrial potential	Active mitochondria in sperm mid-piece are critical for metabolism, membrane function, and motility
Capacitation status	A process of physiological sperm maturation to obtain fertilizing capacity. It happens inside the female reproductive tract, but the capacitation like changes in fresh or cryopreserved sperm are pathological and highly detrimental to the sperm. Therefore, it serves as a negative indicator of sperm fertilizing ability
Apoptosis	Apoptosis maintains and develops the health by eliminating aged and unhealthy cells, but excess apoptosis is detrimental to spermatozoa
Oxidative stress	Reactive oxygen species plays a vital role in sperm physiology, but when it exceeds certain level it becomes the reason for sperm pathology
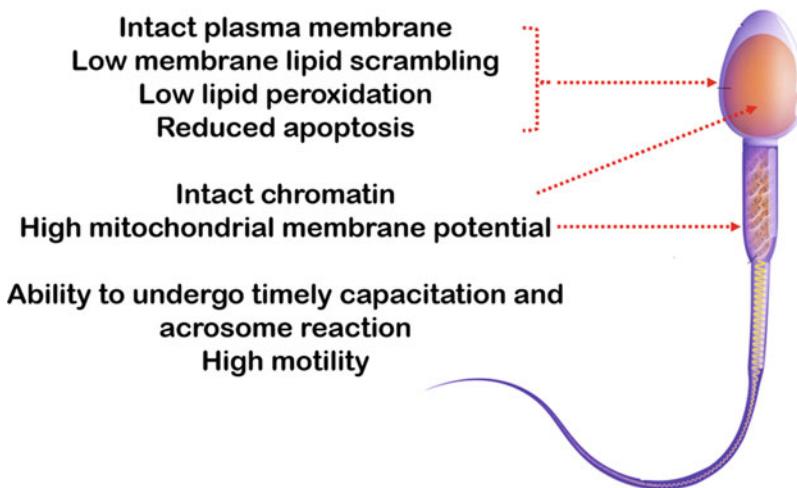
Fig. 14.1 Vital parameters of spermatozoa required for fertilizing an oocyte

sperm which is not enough to predict the fertility of a given semen sample (Rodríguez-Martínez 2003).

There are two reasons for the failure of conventional assays to predict the fertility. First reason is that, spermatozoa are intricate cells and they must possess many characteristics to fertilize their counterpart oocytes (Table 14.1 and Fig. 14.1). However, available assays are assessing only few of the many characteristics a

fertile sperm should encompass. Moreover, the parameters, which are assessed by the conventional assays, are having less correlation with the fertility (Graham 2001). Second reason is that not all the spermatozoa in the ejaculate are having the similar fertilizing capacity. Ejaculate consists of heterogenous, various cohorts of subpopulations as a result of different spermatogenic waves (Rodriguez-Martinez 2006). Hence, evaluating a few numbers of sperm in an ejaculate will not help in predicting fertility.

Therefore, there is an ardent need to adopt a technology that is (1) objective and repeatable in evaluating sperm attributes, (2) evaluates the changes that occur in spermatozoa during cryopreservation like premature capacitation and acrosomal reaction, altered mitochondrial function, reduced motility and DNA damage, alteration in the calcium channels, and capacitation like changes (cryo-capacitation) (Bailey et al. 2003), (3) evaluates multiple attributes of a sperm which include functional attributes which are important for fertility, (4) analyzes large number of spermatozoa in a very short time, and (5) identifies the fertilizing population from a heterogeneous subpopulations of given sample (Peña 2018). The modernization in sperm analytical techniques such as computer-assisted sperm analysis, fluorescent microscopy and flow cytometry, and the arrival of fluorescent probes with the capacity to precisely assess specific sperm functions/phenotypic characteristics fulfilled many of above needs. Predictability of some techniques still remains questionable as they do not have constant higher correlation with fertility; however, they are helpful in predicting low quality semen and in eliminating the infertile semen samples.

14.2 Sperm Kinetics

Sperm acquires motility from epididymis to endure the journey to the site of fertilization (Yoshida et al. 2008). Motility is vital for the sperm to pass through the barriers in female reproductive tract to inhabit fallopian isthmus (Dalton 2011). Thus, we need to understand the sperm movement in the given ejaculate to eliminate the samples, which are having a greater number of sperm that could be resisted by barriers in the female reproductive tract. Commonly, sperm motility is assessed by microscope equipped with phase contrast optics, but the subjective biasness in that method made us to look for an alternative objective method. In that journey, track motility, laser doppler spectroscopy, photometric methods, turbidometry, and computer-assisted sperm analyzer (CASA) were developed (Kathiravan et al. 2011), among which CASA is recognized as a study tool due to its ability to deliver accurate, precise information about sperm concentration, motility, motion kinetics, and morphology by the computer analysis of microcinematography (Lu et al. 2014; Yániz et al. 2015).

CASA can assess the kinetic parameters of a sperm such as Progressive motility (PM), Straight-line velocity (VSL), Average path velocity (VAP), Curvilinear velocity (VCL), Linearity (LIN), Straightness (STR), Amplitude of lateral head displacement (ALH), Beat cross frequency (BCF), and Wobbliness (WOB).

Fig. 14.2 Kinetic parameters of sperm assessed by CASA and their correlation with fertility
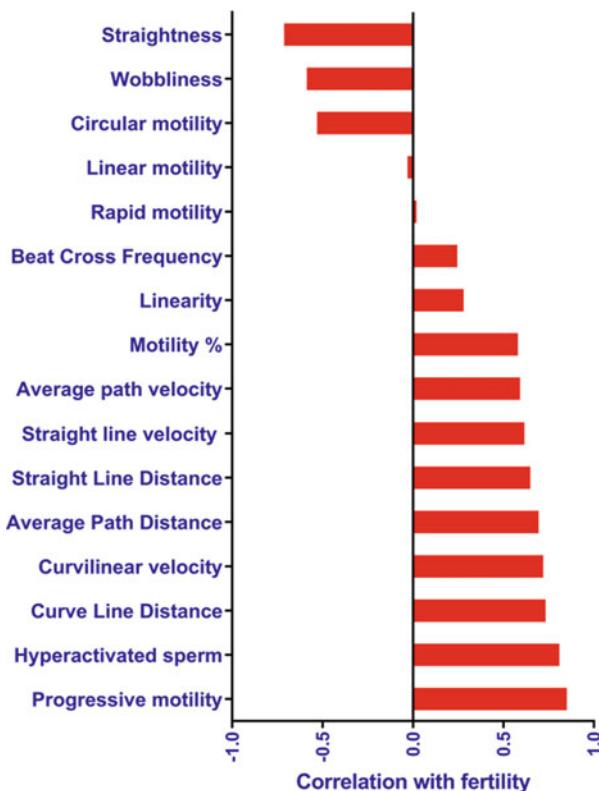
Previous studies reported higher correlation between sperm kinematic parameters (PM, VSL, VAP, and VCL) and bull fertility; 0.63–0.79 (Kasimanickam et al. 2007; Kathiravan et al. 2008; Shojaei et al. 2012; Padrik et al. 2012), 0.62–0.67 (Gillan et al. 2008; Kathiravan et al. 2008; Shojaei et al. 2012), 0.61–0.70 (Kathiravan et al. 2008; Shojaei et al. 2012), and 0.74 (Padrik et al. 2012; Shojaei et al. 2012), respectively. Though it has been shown that CASA-assessed parameters have good correlation with fertility (Fig. 14.2), it is not a sole tool for predicting fertility, because all the sperm with good kinetic properties are not fertile. The modern-day CASA analyzers are equipped with the additional features to perform live/dead sperm count and DNA fragmentation analysis (Amann and Waberski 2014; Mortimer et al. 2015), which could increase the power of CASA in predicting fertility. Sellem et al. (2015) reported that combining CASA and Flow cytometry will help in fair prediction of fertility of bull semen. Padrik et al. (2012) found that the combination of HOST, CASA, and flow cytometry predicted fertility with high accuracy ($r = 0.96$).

14.3 Fluorescence Techniques: The Art of Painting Over the Sperm

The influx of fluorescent probes revolutionized our understanding about sperm. Fluorophores are chemical compounds that can emit light after being excited by the light source. Commonly, emitted light has lower energy and longer wavelength than the absorbed light (stokes shift). Fluorescence occurs when the excited fluorophore emits the photon and comes to lower energy state (Lichtman and Conchello 2005). Nowadays, several options are available to choose fluorophores for assessment of specific sperm attributes (Table 14.2).

Common fluorescence technologies used for semen evaluation includes fluorescent microscopy, confocal microscopy, and flow cytometry. Fluorescent microscope uses laser as a light source to excite the fluorophore, which in turn emits a light with longer wavelength and lower energy to produce magnified image. Confocal microscopy or confocal laser scanning microscopy (CLSM) is advantageous over traditional microscopes by its ability to acquire high optical resolution 3D images of live or fixed sperm labelled by one or more fluorophores by taking images at various depths point by point and by eliminating out-of-focus light (Kim et al. 2017). In both of these microscopes, sperm labelled with fluorescent probes are excited by lasers and the resultant fluorescents are detected, but in confocal microscopy, sample is excited in specific point and then scanned in the entire volume (Pawley 2006). Even though microscopic techniques are having the problem of subjectivity, above-mentioned two microscopes help to understand multiple properties of an individual spermatozoon by the use of different fluorophore combinations.

Table 14.2 Fluorescent probes used (either alone or in combination) in the evaluation of sperm attributes

Attributes	Probes
Plasma membrane integrity (or) viability	SYBR14, PI, CFDA, YO-PRO-1, Hoechst 33258, 7-AAD, Ethidium Homodimer, Ethidium bromide, TO-PRO-3
Acrosome reaction	Lysotracker green, lectins (PSA, PNA, ConA, UEA, and WGA) tagged with FITC, PE, Alexa flour 488, Alexa flour 647, or TRITC
DNA integrity	Chromomycin A3, Acridine Orange
Capacitation changes	CTC, Fluo-3, Fluo-4, Indo-1, Mercyanine540, eFluor 514 calcium sensor, Rhod5, Fura2, BCECF AM, SNARF-1
Mitochondrial status	JC-1, JC-9, JC-10, rhodamine 123, DiOC6, TMRE, TMRM, Mitotracker green, MitoTracker deep red, MitoTracker red, MitoTracker red CMXRos, MitoTracker Orange CMH2TMRos, RedoxSensor red CC-1, Nonyl Acridine Orange, Mito-ID red, MitoFluor green, MitoFluor red 589
Apoptosis	Annexin V & PI assay, YO-PRO-1, SYTO16, Carboxyfluorescein-tagged caspase inhibitor probe
Lipid peroxidation and ROS	BODIPY, MitoSOX, Dihydroethidium, H2DCFDA, DAF FM and DAF-FM diacetate, Monobromobimane, 5-iodoacetamidofluoresceine (5-IAF)

Two types of flow cytometers are available, viz., analytical flow cytometer and fluorescence-activated cell sorter (FACS). The functions and capabilities of analytical flow cytometer and FACS are same except that the latter is equipped to sort the cells. Flow cytometer measures physical and multi fluorescence properties of the cells while they are in a moving fluid stream (Principle of hydrodynamic focusing). Flow cytometry needs a fluorophore-labelled sperm, which will be taken inside the tubular system of an instrument, where the sperm cells are then exposed to laser, one by one (individual files). The resultant emission from the cells is received by different detectors and then the light signals are digitized and software-handled to deliver comprehensible results (Silva and Gadella 2006; Hossain et al. 2011). The excitation and emission wavelength of different fluorophores used for sperm evaluation are shown in Table 14.3. Among the available technologies, flow cytometer possesses several unique characteristics to become a sturdy tool for semen evaluation. It includes high objectivity, repeatability (Graham 2001), multiparametric ability to detect more than one attribute of a single sperm by the simultaneous assessment of multiple fluorochromes attached to the spermatozoa (Gillan et al. 2005; Kumaresan et al. 2017), and ability to analyze large number of spermatozoa in a very short time, even up to 1 lakh cells within a minute (Peña 2018), which is substantially higher than the cells generally observed by microscopic analysis. However, flow cytometric evaluation is not a "gold standard test" for semen evaluation, but it is faraway better than other semen evaluation techniques and provides newer insights. In future, flow cytometry will get limelight and it will revolutionize our understanding on sperm to become the indispensable cornerstone for semen evaluation. Following are some of the important sperm functional attributes that can be assessed by fluorescent technologies using different modern era fluorescent probes.

14.4   Plasma Membrane Integrity

Plasma membrane covering the spermatozoa is directly or indirectly linked to the several functions such as capacitation, acrosome reaction, and the metabolism to preserve motility and homeostasis. Intact plasma membrane is required for the sperm to interact with the epithelial membrane of female reproductive tract and to interact with the oocyte (Rodríguez-Martínez 2003). By evaluating the proportion of plasma membrane intact (viable) and non-intact (nonviable) sperm in the given sample, we could take a decision on increasing the sperm dose for insemination, as viability is considered as a compensable trait (DeJarnette 2005; Dalton 2011). In a cryodamage point of view, we could get an impression about membrane permeability, fluidity, and lipid membrane scrambling (Bailey et al. 2000). A single fluorescent probe or in combination can be used for evaluating membrane integrity. Two types of probes are being used for this purpose—(1) membrane permeable probes (permeable to all nucleated cells—both live and dead) and (2) membrane impermeable probes (normally don't cross the cell membrane, but permeate only when the membrane is damaged). Previously, membrane impermeable probes like propidium iodide (PI) or

Table 14.3 Excitation and emission wavelength of fluorophores used in sperm evaluation

Probes	Excitation wavelength (nm)	Emission wavelength (nm)
Plasma membrane integrity		
SYBR14	516	617
CFDA	494	521
Hoechst 33342	350	461
Hoechst 33258	350	461
PI	493	636
7-AAD	488	647
TO-PRO-3	642	661
Ethidium bromide	300, 360	590
Ethidium homodimer-1	528	617
Acrosomal integrity		
FITC	495	519
TRITC	544	570
PE	496, 546,565	578
Alexa Flour 488	490	525
Alexa Flour 647	650	671
Lysotracker Green	504	511
DNA integrity		
Acridine orange	500 (DNA), 460 (RNA)	526 (DNA), 650 (RNA)
Chromomycin A3	445	575
Mitochondrial integrity		
Rhodamine 123	511 (ethanol), 488 (PBS)	534 (ethanol), 515–575 (PBS)
JC-1	498 (monomer), 585 (aggregate)	530 (monomer), 595 (aggregate)
JC-9	506	525
JC-10	485 (monomer), 535 (aggregate)	535 (monomer), 590 (aggregate)
DiOC6	484	501
TMRE	549	574
TMRM	552	574
MitoTracker Green FM	490	516
MitoTracker Red FM	581	644
MitoTracker Red CMXRos	579	599
MitoTracker Deep Red FM	644	665
MitoTracker Orange CMH2TMRos	554	576
RedoxSensor Red CC-1	540	600
Nonyl Acridine Orange	495	519
Mito-ID Red	558	690
MitoFluor Green	490	516
MitoFluor Red 589	588	622

(continued)

Table 14.3 (continued)

Probes	Excitation wavelength (nm)	Emission wavelength (nm)
Capacitation status		
CTC	392	536
Merocyanine 540	540	624 (tightly packed lipids), 594 (loosely packed)
Fluo3	506	526
Fluo4	494	516
Indo-1	346 (low ca2+), 330 (high ca2+)	475 (low ca2+), 401 (high ca2+)
Rhod5	551	576
Fura2	363 (low ca2+), 335 (high ca2+)	512 (low ca2+), 505 (high ca2+)
eFluor 514 calcium sensor	490	514
BCECF AM	482 (low pH), 503 (high pH)	520 (low pH), 528 (high pH)
SNARF-1	548 (low pH), 576 (high pH)	587 (low pH), 635 (high pH)
Apoptosis		
FITC-tagged Annexin V	495	519
Yo-Pro 1	491	509
SYTO 16	494	515
Carboxy fluorescein-tagged caspase inhibitor probe	490	520
Oxidative stress and lipid peroxidation		
H_2DCFDA	495	527
DHE	518	606
MitoSOX red	510	580
DAF-FM	495	515
DAF-FM diacetate	495	515
BODIPY 581/591	581 (nonoxidized), 500 (oxidized)	591 (nonoxidized), 510 (oxidized)
BODIPY 665//676	665	676
Bis-BODIPY-FL	488	530
Monobromobimane	394	490
5-iodoacetamidofluoresceine (5-IAF)	498	517

ethidium homodimer (EH) alone was used, but nowadays the combination of permeable (i.e., SYBR-14, CFDA) and impermeable probe (i.e., Propidium Iodide, Ethidium homodimer, ethidium bromide) is used (Martínez-Pastor et al. 2010; Hossain et al. 2011).

Using these combinations, moribund sperm population can also be identified. Popularly, SYBR14 + PI combinations are used worldwide, in which, SYBR14 is permeable to all the nucleated cells and stains their nucleus as green, irrespective of
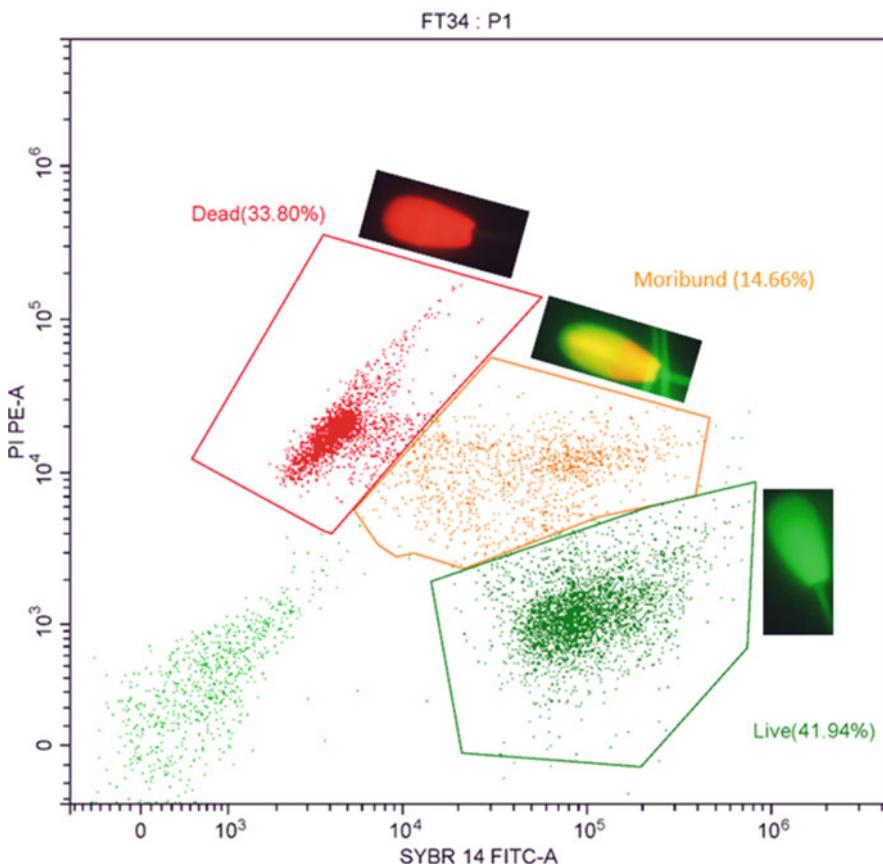
Fig. 14.3 Flow cytometric plot of SYBR-14 + PI staining for viability

their membrane status. Propidium iodide penetrates the membrane-disrupted cells and stains their DNA as red after displacing green color given by SYBR-14. Viability detected by the SYBR14 + PI dyes is highly correlated ($r = 0.64$ to 0.83) with fertility (Januskauskas et al. 2000; Kasimanickam et al. 2007). Next to SYBR14 + PI, CFDA+PI combinations are widely used, in which CFDA is nonfluorescent dye, but after being trapped inside the cells having intact membranes, CFDA is hydrolyzed by an esterase enzyme and releases free 6-Carboxyfluorescein, which results in a fluorescing cell (Hossain et al. 2011). In both of these combinations, live sperm (membrane intact) fluoresce green and dead sperm (membrane damaged) fluoresce red, whereas moribund sperm fluoresce both green and red. Representative subpopulations and their Flow cytometry plots are given in Fig. 14.3. Even though these probe combinations are giving laudable results, their emission wavelengths (SYBR14–617 nm, PI–636 nm) are sometimes prone for spectral overlap; therefore, probes like TO-PRO-3 (emission at 661 nm) and 7-AAD (emission at 647 nm) are emerging as a modern viability probe

(Perticarari et al. 2007; Domınguez-Rebolledo et al. 2010), as they won't interfere with green or orange dyes. From conventional (eosin-nigrosin) to modern (propidium iodide, Hoechst dyes), many of the viability stains are DNA-specific. Therefore, if a sperm is stained by these stains, it reflects the integrity of the plasma membrane covering sperm head only. The integrity of membrane covering the tail can be assessed by Hypoosmotic swelling test – HOST (Colenbrander et al. 2003).

14.5 Acrosomal Integrity

Multiple fusion between plasma membrane and outer acrosomal membrane and exocytosis of acrosome vesicular content is a prerequisite for the sperm–oocyte interaction and hence fertilization. Poorly formed acrosome or premature loss of acrosome can compromise the fertility (Abou-Haila and Tulsiani 2000; Gerton 2002). Cryopreservation is a major cause of damages to acrosome and changes the intracellular calcium concentrations to induce premature acrosome reaction (Bailey et al. 2000). The lectins used for assessing acrosome integrity includes PNA [Peanut (*Arachis hypogaea*) agglutinin], PSA [(Pea (*Pisum sativum)* agglutinin], ConA (Concanavalin A), UEA (Ulex europaeus agglutinin), and WGA (*Triticum vulgaris* agglutinin) (Gillan et al. 2005; Hossain et al. 2011). These lectins are commonly tagged with either of FITC, Phycoerythrin (PE) (Nagy et al. 2003), Alexa Flour 488 (Alvarez-Rodriguez et al. 2018), Alexa Flour 647 (Murphy et al. 2017), or TRITC (Domınguez-Rebolledo et al. 2010).

Among these lectins, PNA and PSA are commonly used, in which PNA has an affinity towards β galactose of the outer acrosomal membrane, whereas PSA has an affinity towards α-galactose and α-mannose of the acrosomal matrix. PNA has advantage over PSA, as PSA has a nonspecific binding with egg yolk particles and other parts of the spermatozoon such as head and tail (Gillan et al. 2005; Hossain et al. 2011). Therefore, while assessing the semen extended with egg yolk media, PNA should be used or the egg yolk particles need to be gated out using DNA binding probes. The commonly used combinations include FITC-conjugated PNA or PSA with propidium iodide to evaluate acrosome integrity and viability contemporaneously. Representative subpopulations and their Flow cytometry plots are given in Fig. 14.4. By using this combination, Singh et al. (2016) reported positive correlation of live acrosome intact (0.59) and negative correlation of dead acrosome reacted (−0.45) with the buffalo bull fertility. Triple combinations such as Phycoerythrin (PE)-tagged PNA, SYBR14, PI (Nagy et al. 2003), and TRITC-conjugated PNA, YoPro1, Mitotracker deep red (Domınguez-Rebolledo et al. 2010) have also been proposed. Other methods such as Fluorescent-conjugated anti CD46 antibody (Grunewald et al. 2006), anti-acrosin antibody (Thomas et al. 1997), anti-hyaluronidase antibody (Baccetti et al. 1999), and acidotrophic organelle-specific probe like LysoTracker (Harper et al. 2006) are also used to evaluate acrosome integrity.
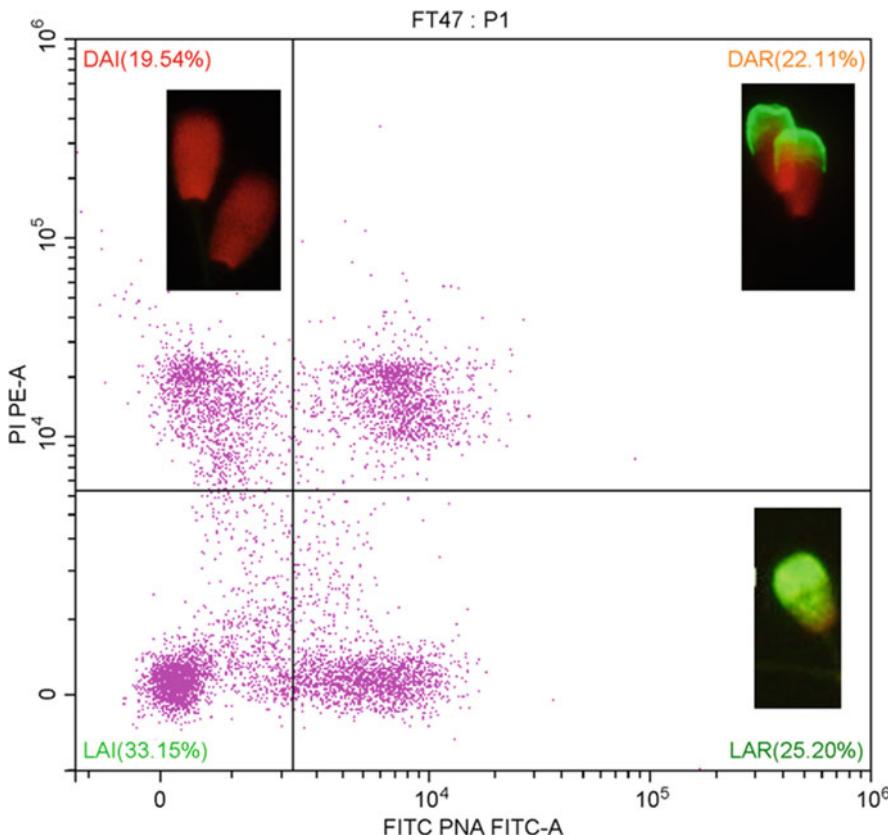
Fig. 14.4 Flow cytometric plot of FITC-PNA + PI staining for acrosomal integrity (lower left quadrant—live acrosome intact; lower right quadrant—live acrosome reacted; upper right quadrant—dead acrosome reacted; upper left quadrant—dead acrosome intact)

14.6 DNA Integrity

Sperm DNA ensures the transfer of tightly packed genetic information to the oocyte (Agarwal and Allamaneni 2005). DNA integrity is important for the sperm to initiate and sustain fertilization, embryonic development, and for offspring fitness (Saacke 2008; Martínez-Pastor et al. 2010). The DNA integrity of sperm can be assessed by Sperm Chromatin Structure Assay (SCSA), Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay, Chromomycin A3 staining (CMA3), Sperm Chromatin Dispersion test (SCD), COMET (Single cell gel assay) assay, In situ nick translation, 8-oxo-7,8 dihydro-2 deoxyguanosine (8-OH-Dg), Toluidine blue staining, Acridine Orange test (AOT), and Sperm chromatin decondensation (Sharma et al. 2004), among which SCSA, TUNEL, and CMA3 became popular worldwide. SCSA measures the susceptibility of sperm DNA to the

denaturation in situ, which is induced by low pH treatment. SCSA uses acridine orange as the probe, which can bind to intact DNA to fluoresce green (dsDNA) and can bind with the DNA strands at the sites of breaks to fluoresce red (ssDNA) (Evenson and Wixon 2006; Evenson 2013). In SCSA, DNA Fragmentation Index (DFI) or Cell out of the main population (COMP) is measured by the ratio of red fluorescence/total fluorescence. Cut-off value of DFI (Table 14.4) in bulls is 20%, in boars 17.6%, and in human 27% (Ballachey et al. 1988; Rybar et al. 2004; Niu et al. 2011). DFI is negatively correlated (-0.50 to -0.87) with bull fertility (Bochenek et al. 2001; Januskauskas et al. 2001, 2003; Evenson and Wixon 2006; Kasimanickam et al. 2007; Kumaresan et al. 2017). Easy, objective, and rapid evaluation of higher number of sperm and simultaneous assessment of protamine deficiency (by analyzing high DNA stainability) makes the SCSA as an assay of choice for the evaluation of DNA integrity. Representative photograph of sperm DNA integrity assessment using acridine orange is given in Fig. 14.5.

TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate (dUTP) nick end labeling) assay targets the 3'hydroxyl ends resulted from single stranded or double stranded breaks and labels it with the FITC-tagged dUTP by the help of TdT (Domínguez-Fandos et al. 2007). TUNEL assay has the advantage over SCSA, as the former measures the actual DNA damage and the latter measures the susceptibility of the DNA to external damaging conditions by inducing it using low pH treatment. However, the expensiveness and lengthy tiresome procedures involved in TUNEL assay make it as a second option (Sharma et al. 2013). The replacement of histones by the protamine in the sperm chromatin during spermatogenesis ensures the safe packaging of genetic information and tightly packed chromatin (Balhorn 2007). Fertilizing potential of the sperm with deficient protamine or excessive histones (can be assessed by aniline blue staining) is compromised. Evaluating the protamine-deficient sperm with loosely packed chromatin is possible by means of Chromomycin A3 (CMA3) staining. The results of CMA3 staining are correlated with the DNA breaks (Manicardi et al. 1995) and fertility (-0.37; Singh et al. 2016). High DNA stainability % (HDS), which is estimated by SCSA is the indicator of sperm with protamine deficiency. Cut-off of HDS in men and bull is 15% and 3.5%, respectively (Rybar et al. 2004; Niu et al. 2011). For a recent review on sperm DNA integrity and male fertility in farm animals, the readers may refer to the article by Kumaresan et al. (2020).

14.7 Mitochondrial Integrity

Importance of mitochondria in sperm physiology is to produce ATP by oxidative phosphorylation for the metabolism, membrane function, and sperm motility. Integrity and normal functionality of the mitochondria distributed in the mid-piece of the tail are an indicator of sperm health (Hu et al. 2017; Moraes and Meyers 2018). Rhodamine 123, a probe that stains the mitochondria of living cells, was the classical probe used previously to assess mitochondrial integrity. However, JC-1 (5, 5', 6, 6'-tetrachloro 1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine Iodide) and

Table 14.4 Cut-off Values reported for DNA damage in sperm in relation to sperm fertility

DNA damage %	Fertility evaluated as	Species	Authors
SCSA			
27	BP CP IR after IVF ICSI	Human	Boe-Hansen et al. (2006)
27	CP after IVF ICSI	Human	Larson-Cook et al. (2003)
27	BP CP after IVF ICSI	Human	Bungum et al. (2004)
30	BP CP PL after IUI IVF ICSI	Human	Bungum et al. (2007)
9–27	CP PL after IVF ICSI	Human	Lin et al. (2008)
15	CP after ICSI	Human	Micinski et al. (2009)
27	CP PL after IVF	Human	Niu et al. (2011)
15–30	CP PL after ICSI	Human	Zini et al. (2005)
15–50	CP after ICSI	Human	Dar et al. (2013)
19–30	CP PL after IVF ICSI	Human	Speyer et al. (2010)
18.9	CP after intercourse	Human	Ribas-Maynou et al. (2013)
30	CP after IVF ICSI	Human	Jiang et al. (2011)
25	BP CP PL after ICSI	Human	Yang et al. (2013)
40	Fecundability after intercourse	Human	Spano et al. (2000)
30	CP BP after intercourse	Human	Evenson et al. (1999)
30	CP after IVF and ICSI	Human	Evenson and Jost (2000)
30.28	Retrospective	Human	Venkatesh et al. (2011)
20	Competitive index after Heterospermic insemination	Bull	Ballachey et al. (1988)
20.8	Pregnancy rate 90 d after AI	Bull	Rybar et al. (2004)
TUNEL			
20	CP after intercourse	Human	Ribas-Maynou et al. (2013)
15	CP PL after IVF ICSI	Human	Benchaib et al. (2007)
36.5	CP after IVF	Human	Henkel et al. (2003)
24.3	CP after ICSI	Human	Henkel et al. (2003)
4	CP after IVF	Human	Sun et al. (1997)
19.25	Retrospective	Human	Sharma et al. (2010)
20	Retrospective	Human	Sergerie et al. (2005)

AI artificial insemination, *BP* biochemical pregnancy, *CP* clinical pregnancy, *PL* pregnancy loss, *ICSI* intra cytoplasmic sperm injection, *IUI* intrauterine insemination, *IVF* in vitro fertilization

mitotracker dyes emerged as a modern probe due to their ability to retain in the cells and the ability to assess mitochondrial membrane potential (MMP), which are lacking in R123 (Cottet-Rousselle et al. 2011). JC-1 is a lipophilic, cationic, and carbocyanine stain. Spermatozoa with actively functioning mitochondria accumulate more amounts of JC-1 compared to poorly functioning mitochondria. At low
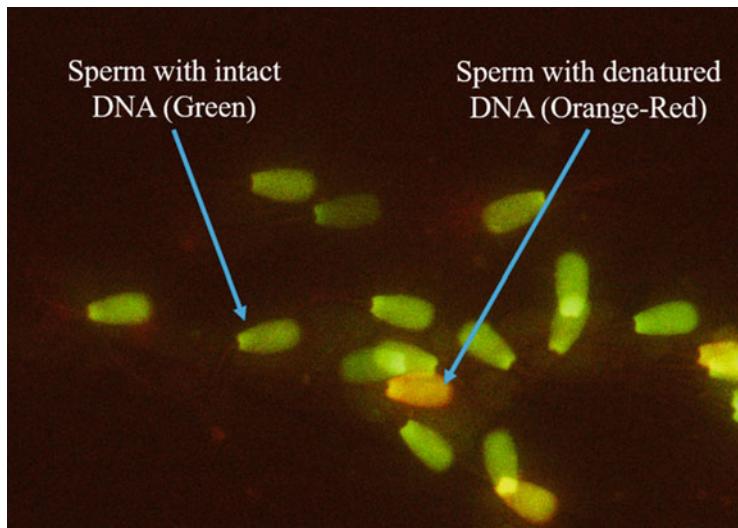
Fig. 14.5 Fluorescent microscopic picture of sperm with intact and damaged DNA

concentrations, JC-1 exists as a monomer and fluoresces green, but at higher concentrations, it forms aggregates and fluoresces red-orange (Gravance et al. 2000; Moce and Graham 2008). Therefore, two populations of sperm, viz., sperm with high mitochondrial membrane potential (MMP) as Red-orange and low MMP as green, can be visualized. Representative photograph of sperm mitochondrial membrane potential assessment using JC-1 and its Flow cytometry plots are given in Fig. 14.6. Variants of JC-1 such as JC-9 and JC-10 have also been developed. Green fluorescence of JC-9 doesn't change based on membrane potential, but its red fluorescence rises when the membrane is hyperpolarized (Cottet-Rousselle et al. 2011). JC-10 is a superior alternative to JC-1, especially in terms of water solubility (Elkhawagah et al. 2020). As like JC probes, DiOC6 is also a carbocyanine dye which is efficiently used with PI in boar sperm (Kumaresan et al. 2009a, 2009b).

Other stains that can be used to assess mitochondrial functionality include TMRE, TMRM, RedoxSensor Red CC-1, Nonyl Acridine Orange (NAO), Mito-ID Red, MitoFluor probes (MitoFluor Green, MitoFluor Red 589), and MitoTracker probes (MitoTracker Green, MitoTracker Red, MitoTracker Deep Red, MitoTracker Red CMXRos, and MitoTracker Orange CMH2TMRos) (Srivastava and Pande 2016; Boe-Hansen and Satake 2019). However, many comparative studies found that JC-1 is a mitochondria-specific highly sensitive stain while comparing it with rhodamine123, Mitotracker Deep Red, DiOC6, TMRE, and CMX-Ros (Garner et al. 1997; Salvioli et al. 1997; Marchetti et al. 2004). Many of the common probes emit green or red light, therefore JC-1 can't be combined with majority of the other probes to evaluate any extra parameter simultaneously. In contrast to JC-1, the availability of mitotracker dyes in a wide range of fluorescence emission enables them to be combined with other probes to study multiple parameters. By using only one
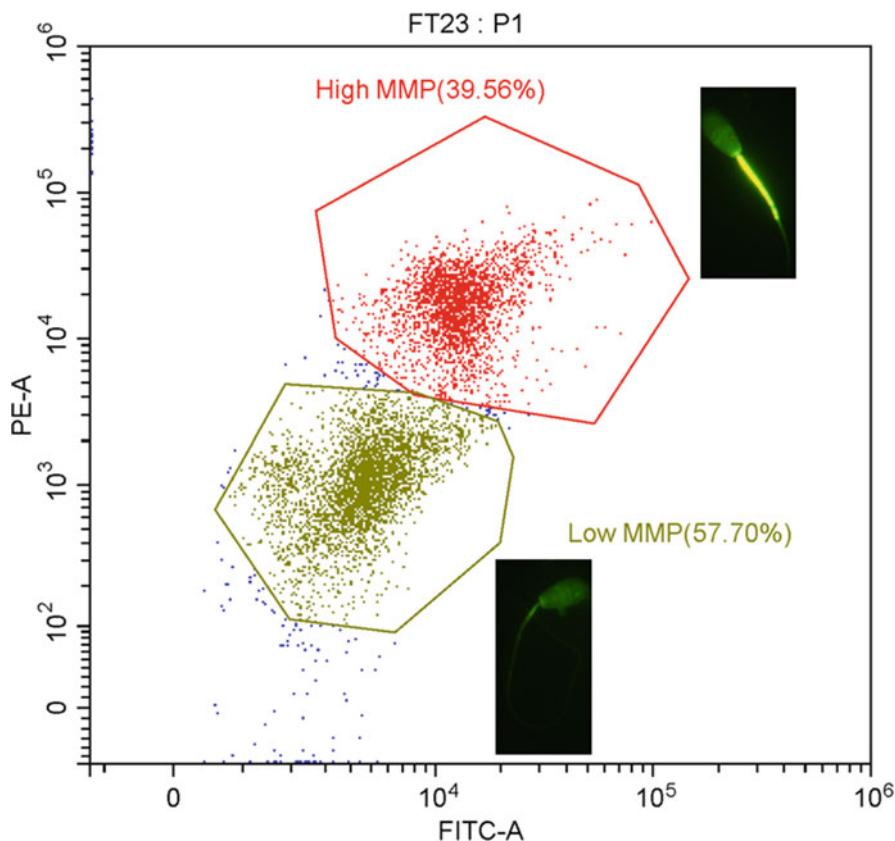
Fig. 14.6 Flow cytometric plot of sperm with high and low mitochondrial membrane potential (MMP)

detector, MitoTracker Deep Red can detect high and low MMP after being excited with red laser. JC-1-stained bull spermatozoa are highly correlated with the motility ($r > 0.96$; Garner et al. 1997) and viability which is estimated by SYBR14/PI ($r > 0.9926$; Huo et al. 2002). Antibodies against Cytochrome C Oxidase (COX 1) have also been used to assess the mitochondrial functions by fluorescent microscopy (Amaral et al. 2007).

14.8 Capacitation Status

Capacitation is the penultimate process in the sperm maturation and a prerequisite for the sperm to obtain fertilizing capacity. It is physiological when this process happens inside the female reproductive tract, but the capacitation like changes that are happening during cryopreservation are pathological and highly detrimental to the sperm (Bailey et al. 2003; Ickowicz et al. 2012). Cryopreservation causes the
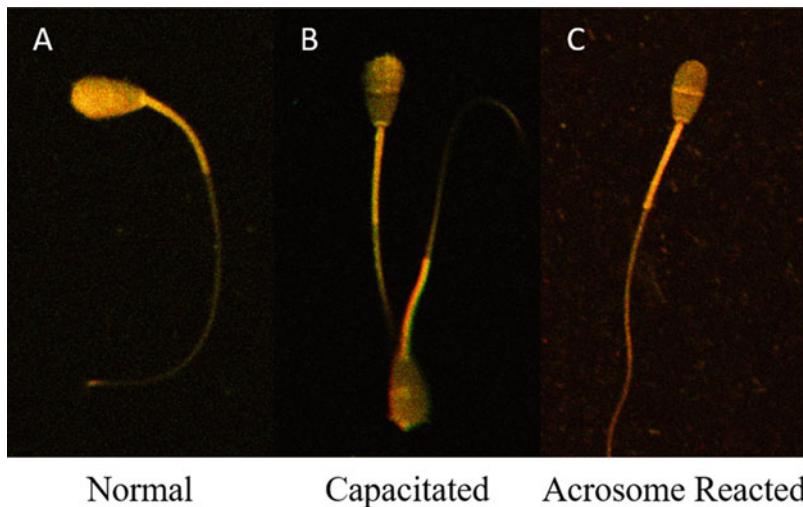
Fig. 14.7 CTC staining shows capacitated and acrosome reacted sperm

structural changes of plasma membrane, altered permeability, influx of calcium, sodium ions, efflux of cholesterol, increased intracellular PH, and protein tyrosine phosphorylation leading to cryo-capacitation. This process risks the life of the sperm by causing sublethal damages and finally compromise the fertility (Bailey et al. 2000, 2003). Therefore, capacitated spermatozoa have a significant negative correlation (−0.52; Januskauskas et al. 2000) with fertility. Commonly, Chlortetracycline (CTC) stain, a calcium chelator that locates calcium, is used to assess uptake of calcium during sperm capacitation (Collin et al. 2000). Representative photograph of sperm capacitation status assessment using CTC is given in Fig. 14.7. However, CTC is not well adopted in flow cytometry, therefore, the limelight turned towards Merocyanine 540 (M540), a lipophilic probe that can assess membrane fluidity. It binds to phospholipid membrane and its fluorescence intensity increases with membrane lipid scrambling (Muratori et al. 2004; Pena et al. 2004). M540 is still used as an earlier marker of capacitation to understand the degree of lipid disordered in the plasma membrane. Annexin V can detect the capacitation by indicating the externalization of phosphatidylserine to the outer leaflet of membrane (Saravia et al. 2007).

Calcium influx and the increase in the intracellular calcium can be detected by Fluo-3 (Piehler et al. 2006), Fluo-4 (O'Rand and Widgren 2012), and Indo-1 (Dubé et al. 2003), among which the need of UV laser to excite the latter makes Fluo stains as preferred. Representative photograph of sperm intracellular calcium status assessment using Fluo-3 and its Flow cytometry plots are given in Fig. 14.8. Other calcium indicators like Fura-2, eFluor 514 calcium sensor (Petrunkina and Harrison 2013), and Rhod5 (Yeste et al. 2015) are less commonly used. Increased intracellular pH during capacitation can be assessed by 2′,7′-bis-(2-carboxyethyl) -5-(and-6) -carboxyfluorescein, acetoxymethyl ester (BCECF AM), and SNARF-1, which get into plasma membrane and fluoresce based on pH (Pons-Rejraji et al. 2009).
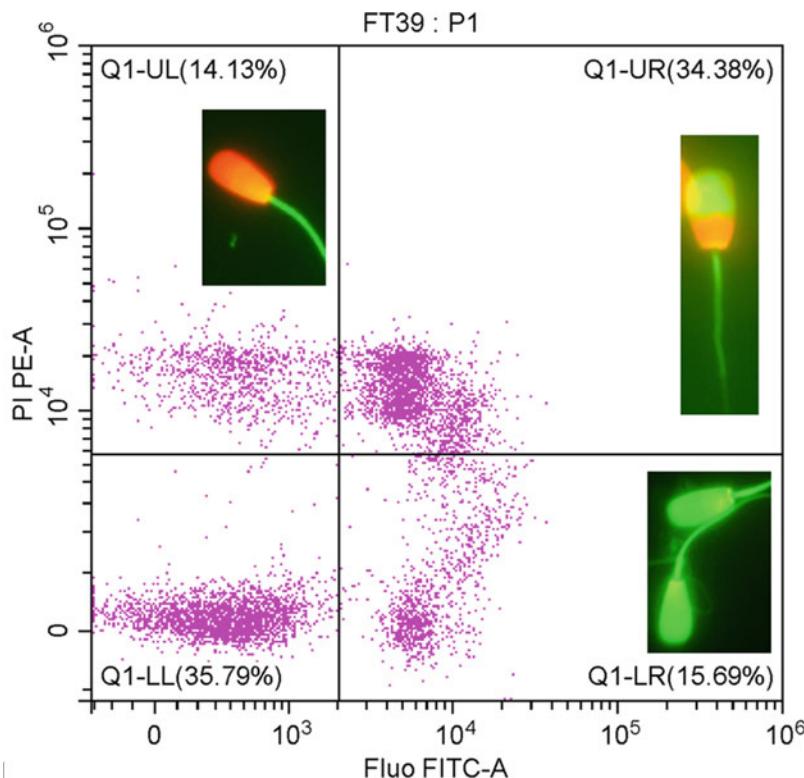
Fig. 14.8 Flow cytometric plot of Fluo-3 staining for intracellular calcium (lower left quadrant—live low calcium; lower right quadrant—live high calcium; upper right quadrant—dead high calcium; upper left quadrant—dead low calcium)

Evaluating the protein tyrosine phosphorylation that happens during capacitation by using antiphosphotyrosine antibody in flow cytometry is emerging as an easy alternative to western blotting (Vignesh et al. 2020).

14.9 Apoptosis

Apoptosis (i.e., falling of leaves from trees) supports the maintenance and development of the health by eliminating aged and unhealthy cells. The caspase activation, translocation of phosphatidylserine from cytoplasm to outer plasma membrane, nucleus condensation, fragmentation of DNA, and alteration in MMP are the markers of apoptosis (Martin et al. 2004; Said et al. 2010; Martínez-Pastor et al. 2010; Hossain et al. 2011). Apoptosis also happens in spermatozoa and can compromise the fertilizing potential of the spermatozoa (Kumaresan et al. 2009a, 2009b; Hossain et al. 2011). Among the changes occurring during apoptosis, the translocation of phosphatidylserine (PS) from inner leaflet to the outer leaflet of the sperm
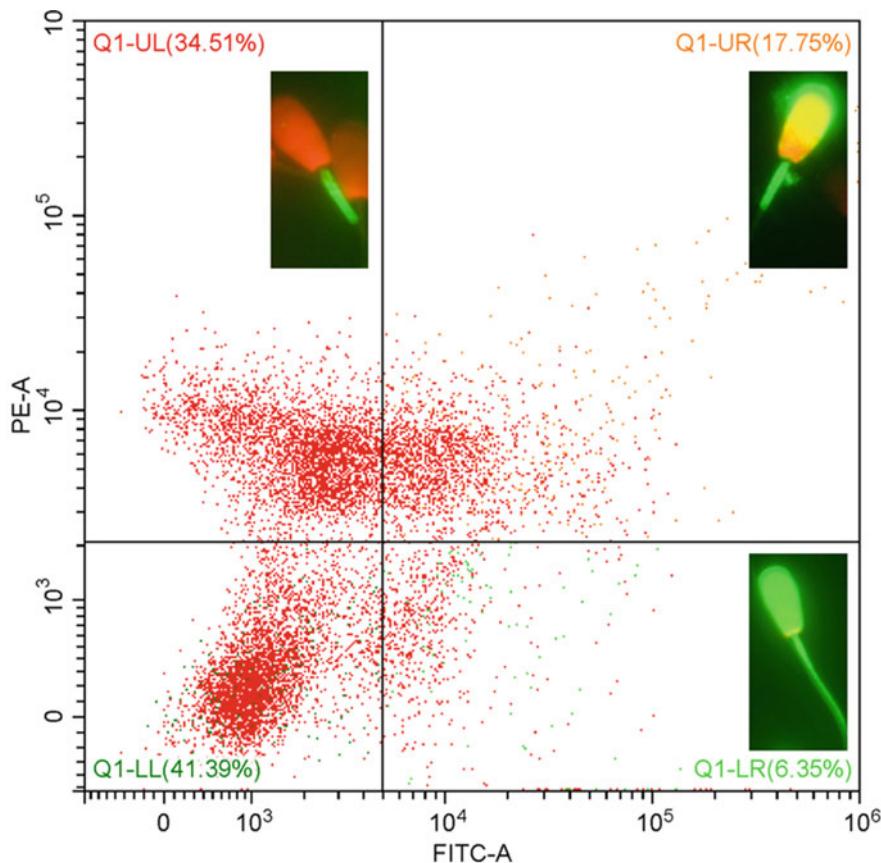
Fig. 14.9 Flow cytometric plot of Annexin-V + PI staining (lower left quadrant—viable; lower right quadrant—apoptotic; upper right quadrant—early necrotic; upper left quadrant- necrotic)

plasma membrane is the earliest sign of apoptosis. Therefore, Annexin V, a calcium-dependent phospholipid binding protein, which can easily locate and bind to the exposed PS with high affinity, became the probe of choice for the detection of sperm apoptosis. Annexin V is usually used in combination with nuclear stains (PI) to simultaneously assess viability. Annexin V – PI assay estimates four different populations of the sperm such as viable, apoptotic, early necrotic, and necrotic (Anzar et al. 2002). Representative photograph of sperm apoptosis status assessment using Annexin V—PI combination and its Flow cytometry plots are given in Fig. 14.9. Apoptotic sperm in the frozen semen of bull and buffalo predicted by Annexin V-PI assay has the correlation of -0.48 and -0.39 with the fertility, respectively (Anzar et al. 2002; Singh et al. 2016). The alteration of membrane permeability by apoptosis can be detected by Yo-Pro1. The apoptotic cells which are permeated by Yo-Pro1 remain impermeable for the PI (Dead cell stain). Yo-Pro1 was used in combination with merocyanine 540 or Hoechst 33342 (Hallap et al.

2006) or Mitotraker deep red (Peña 2015). Other methods to detect the apoptosis include SYTO-16 probe (Perticarari et al. 2007), detecting the caspase (apoptosis-specific cysteine proteases) activation during apoptosis by carboxyfluorescein-tagged caspase inhibitor probe (Paasch et al. 2003), detecting the cleavage of poly (ADP-ribose) polymerase (Jha et al. 2009), and detecting BAX proteins by western blotting (Dogan et al. 2013).

14.10 Oxidative Stress

Reactive oxygen species play a vital role in sperm physiology, but when it exceeds certain range then it becomes the reason for sperm pathology. Oxidative stress occurs when more amount of reactive oxygen species are produced or when the antioxidants are not well enough to scavenge ROS (Agarwal and Allamaneni 2004). Probes that are having the ability to fluoresce during oxidation and accumulate intracellularly can be used for the reactive species detection. Commonly 2′, 7-′-dichlorodihydrofluorescein diacetate—H_2DCFDA (to detect H_2O_2) and dihydroethidium or hydroethidine—DHE (to detect superoxide) are used for this purpose (Awda et al. 2009). The nonfluorescent, cell permeable H_2DCFDA retains inside the cell, after being de-esterified by intracellular esterases, and emits green fluorescence when it is converted to 2′,7′-dichlorofluorescein (DCF) upon the presence of H_2O_2 (Guthrie and Welch 2006; Domınguez-Rebolledo et al. 2010). Studies found that CM-H_2DCFDA has better retention capacity within live cells than Carboxyl H_2DCFDA and H_2DCFDA (Crespo-Félez et al. 2017; Li et al. 2018). Upon the presence of superoxide, DHE oxidizes to form ethidium that becomes fluorescent (Boe-Hansen and Satake 2019). Mitochondrial-specific superoxide in live sperm can be detected by MitoSOX (Koppers et al. 2008). Representative photograph of sperm mitochondrial-specific superoxide status assessment using MitoSox and its Flow cytometry plots are given in Fig. 14.10.

Reactive oxygen species can damage the phospholipids, which is the major factor for the integrity of plasma membrane and mitochondrial sheath. Therefore, lipid peroxidation is negatively correlated with membrane integrity (−0.789; Ferrusola et al. 2009), mitochondrial membrane potential (−0.689; Ferrusola et al. 2009), and bull fertility (−0.78; Kumaresan et al. 2017). In spite of the availability of methods to detect breakdown products of lipid peroxidation such as malondialdehyde (TBARS assay), 4-hydroxynonenal, and acrolein, BODIPY probe-based assay emerged as a sensitive tool. BODIPY probe binds to cell membrane and changes from red (non-peroxidized membrane) to green (peroxidized membrane) as a response to free radical attack on membrane (Brouwers and Gadella 2003). Representative photograph of sperm lipid peroxidation status assessment using BODIPY is given in Fig. 14.11. Different types of BODIPY probes include BODIPY 581/591, BODIPY 665/676, and Bis-BODIPY-FL, in which latter is used to evaluate phospholipase activity (Awda et al. 2009). Reduced thiol concentrations can be assessed by monobromobimane (mBBr; Kumaresan et al. 2017), 5-iodoacetamidofluoresceine (5-IAF), and maleimides (Gadea et al. 2005; Martínez-Pastor et al. 2010).
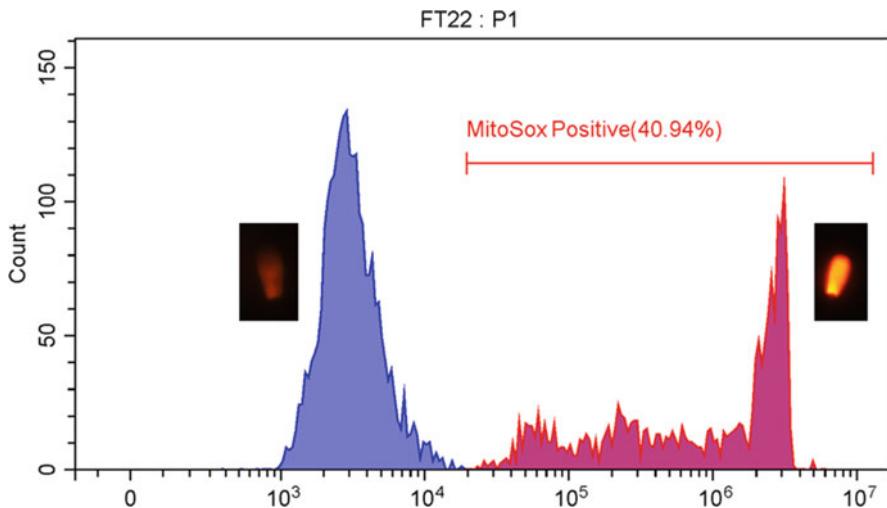
Fig. 14.10 Flow cytometric plot and the representative fluorescent microscopic pictures of MitoSox staining for mitochondrial ROS
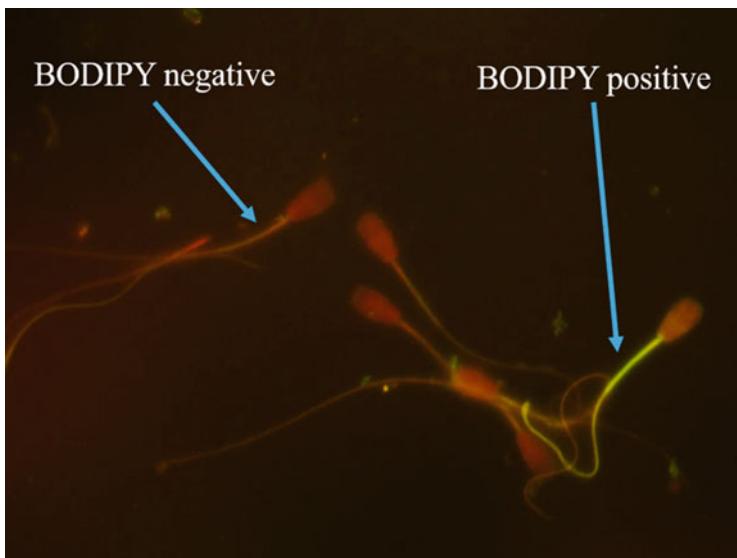

Fig. 14.11 Fluorescent microscopic picture of BODIPY staining for lipid peroxidation

14.11 Perspective and Prospective

Assessment of sperm phenotypic and functional characteristics continues to remain the mainstay of bull fertility prediction. However, till now there is no single test, which can predict the fertility with 100% accuracy. One of the reasons is that the routine semen analysis techniques simply evaluate basic variables of sperm appearance and viability, but cannot disclose subjacent structural and functional details. Since several physiological events precede sperm fertilizing an oocyte, assessment of single parameter cannot predict the sperm fertility. However, performing a battery of tests and combining their results will help us to assess as many as parameters required for the sperm to successfully fertilize the oocyte. It is not possible to perform and use all kind of assays and technologies for predicting fertility. Therefore, we need to find out the combination of assays that are enough to predict fertility with maximal accuracy. Recently, we developed a fertility prediction model (Kumaresan et al. 2017) to differentiate low-and high-fertile cattle bulls with the R^2 of 0.83 [NRR = 55.71 + (0.39 × Live) + (0.37 × Live H_2O_2 negative)— (0.39 × %DFI)] for Holstein Friesian bulls. Similarly, in buffalo bulls when the sperm membrane integrity, acrosomal reaction, apoptosis, protamine deficiency, and membrane scrambling were analyzed separately, the fertility prediction accuracy was not more than 38%; however, when those tests were combined, the prediction accuracy increased to 74%. Among the different combinations evaluated, the proportion of live acrosome intact spermatozoa and spermatozoa with lipid peroxidation showed high correlation with fertility (Singh et al. 2016).

Another obvious reason could be that the most routine semen evaluations include small subsamples of a large, heterogeneous number of spermatozoa in the ejaculate. On an average, one bull ejaculate contains 5 billion spermatozoa. To have an idea about the sperm quality, we assess the quality of spermatozoa in representative samples. In any microscopy, generally 200 number of spermatozoa are analyzed, which is 0.00000004% of total sperm in the ejaculate. Therefore, by analyzing this much minimal proportion, we can't attain complacence in sperm quality analysis because of the presence of different subpopulations of sperm in the same ejaculate which were produced during different spermatogenic cycles. Given the fact that an ejaculate contains different sperm subpopulations, we need to switch to high-throughput assays that can assess several thousands of spermatozoa in shorter time with high repeatability. High-throughput techniques like Flow cytometry can assess at least one lakh sperm per sample, which is 0.00002% of total sperm in the ejaculate. But this technique is expensive and requires skilled manpower. The emergence of multicolor experiments in flow cytometry paved the way to assess many attributes of the sperm simultaneously. In future, the discovery of new probes with varying emission range without spectral overlap to access different attributes of a single spermatozoon may enable us to predict the fertility of the sample by single assay. The recent CASA machines are coming with the ability to assess not only kinetics, but also viability and DNA integrity. Flow cytometric evaluation of ejaculate after improved sperm selection techniques and combining the results of flow cytometry with in vitro function tests and kinetic parameters may help us in

predicting the fertility of given sample. Ongoing researches in flow cytometry may bring the analyzer specific sperm orientation and real time imaging of every spermatozoon while crossing laser and simplified analysis. In future, flow cytometry-based high-throughput techniques could become an indispensable tool for semen evaluation to revolutionize our understanding on sperm physiology and fertility.

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β-Defensins: Antimicrobial Peptides at the Intersection of Immunity and Male Fertility

15

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Abstract

The mammalian spermatozoon is a consequence of extensive and intricate bio-chemical, physiological, and morphological cellular differentiation events which take place in the testes during a spermatozoon's development. However, the testicular still requires distinct post-gonadal modification events in the various sperm surface biomolecules to become competent to fertilize the oocyte. The chronological interactions of the sperm occurring sequentially with the surrounding medium of the three distinct epididymal regions are believed to conclude the final steps of spermatogenesis. A number of secretagogues, e.g., the highly glycosylated and negatively charged, cysteine-rich antimicrobial peptides, β-defensins (BDs), are applied onto sperm surface which assist the spermatozoa in survival during its tortuous journey in the female reproductive tract (FRT) and are added on spermatozoa surface. Hitherto thought of as effectors of the innate immune system, recent research has revealed multiple and potentially epistatic roles of these proteins which possess the widest taxo-nomical distribution. Their role in reproduction especially in male fertility has gained considerable attention in the last two decades. The BDs have crucial roles to play in sperm cervical mucus penetration, capacitation, and zona-recognition. Furthermore, these multifunctional molecules "cross talk" with the adaptive immune system and can modulate the host's immune-competent cell responses, e.g., those mounted against the spermatozoa in the FRT. Overall, these AMPs constitute an important, nonspecific component of the innate immunity in animals

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and humans with potential reproductive immunobiological roles in multiple mammalian species including the ruminants.

The antagonistic relationship between fertility and production can be explained by the 'trade-off' hypothesis proposed in the context of evolutionary biology which signifies that selection for production traits is often accompanied by a concurrent decrease in fertility (Berry et al. 2014, 2016). The augmented prioritization of nutrients and energy for increased yields is thus associated with a drop in fertility. Moreover, it is now widely accepted that the selection for male fertility is complicated because of the lower heritability of fertility traits in comparison to the production traits. The low conception rate (CR) in farm animals like buffalo causes an economic burden especially on the dairy sector and farmers in agriculture-based economies. To overcome the limitation of poor CRs in bovine animals, immense interest has spawned in devising breeding strategies including the methods to augment the production of quality semen and to best utilize the available semen (Srivastava and Kumaresan 2014; Anonymous n.d.; Huang et al. 2010). There is a pressing need for exploration of the factors that govern successful fertilization and its regulation in the bovine model. The immune system is known to play a crucial role in fertilization and pregnancy establishment (Young 2016). The innate host defense peptides (HDPs), beta-defensins (BDs), appear to be one such factor now implicated in the survival and performance of the spermatozoa in the FRT. Recent reports have correlated the expression of a unique class of these antimicrobial peptides referred to as class-A beta-defensins (CA-BD) with sperm maturation, function, and fertility in many mammalian species. Despite their importance, the molecular functions and biological roles of only a limited number of BDs have been studied vis-à-vis their role in reproductive physiology. It is, consequently, very much essential to elucidate and understand how these pleiotropic molecules (BDs) assist the spermatozoa in the intricate process of fertilization in bovine animals.

15.1   The Tailoring of Sperm Surface in the MRT

The recognition of self and non-self is the keystone of defending the body against any pathogenic or allogenic substances/organisms. However, the female not only tolerates the allogenic male gametes, but also assists them in their journey towards the ovum, which is indeed essential for the continuation of species (Guerin et al. 2009). The greatest mystery surrounding fertilization is the mechanism by which the allogenic spermatozoa evade the immune responses, camouflaging themselves as a friend rather than foe in the hostile immunologic milieu of the female reproductive tract (FRT). Since adaptive immunity is known to be largely absent in the male reproductive tract (MRT), the male host must have evolved effective mechanisms for

the protection of spermatozoa. The Sperm Surface Remodelling (SSR) events that occur during the transit of nascent spermatozoa through the epididymal lumen are known to confer protection and functional and survival abilities to the spermatozoa in the FRT (Hall and Killian 1987; Jones 1998; Gadella and Boerke 2016). The epididymis can be broadly subdivided into three segments, viz., the caput, corpus, and cauda. These regional segments differ in their morphology, function, and luminal composition, and therefore, present distinct and unique patterns of gene expression and hence the protein abundance (Johnston et al. 2005; Jelinsky et al. 2007; Belleannée et al. 2012). Disparate secretagogues present in the lumen of these regional segments are added onto the surface of traversing spermatozoa which are known to interact with the extracellular luminal milieu of the epididymis. These subtle interactions are known to modify the sperm surface in a series of sequential biochemical modifications (Ribeiro et al. 2016; Belleannée et al. 2011a, b; Robaire and Hinton 2015). These radical changes in the sperm surface are thus crucial not merely for surmounting the various physiochemical and immunological barriers in the FRT, but also for the molecular mechanisms responsible for sperm-egg interactions (Tollner et al. 2012; Fernandez-Fuertes et al. 2018). Several surface proteins, lipids, and glycans are either removed or modified and several novel antigens are either adsorbed or inserted in the spermatozoa plasma membrane in addition to changes in their localization (Guyonnet et al. 2011; Belleannée et al. 2011a, b; Dacheux et al. 2009). These antigens commonly include— (1) the epididymal coat proteins (ECPs) which may be deep-seated in the plasma membrane via the hydrophobic interactions, (2) electrostatically bound proteins, (3) Glycosyl-phosphatidylinositol (GPI)-anchored proteins and proteins interacting with them, and (4) proteins in apical blebs known as epididymosomes that are embedded in the sperm plasma membrane. The major events occurring during epididymal SSR thus comprise of: (a) enzymatic processing of the membrane-associated proteins; (b) variations in the composition of membrane-lipids; (c) changes in the glycoconjugates (GCs) associated with the sperm glycocalyx; and (d) adsorption, insertion, modification, and binding of (glyco) proteins (Guyonnet et al. 2011; Skerget et al. 2015; Aitken et al. 2007). The glycosylated proteins acquired by the spermatozoa during their transit in the epididymis and pre-ejaculatory ducts (Fig. 15.1) are known to play crucial roles in successful fertilization (Tollner et al. 2012; Flickinger et al. 1990; Rooney et al. 1993; Yeung et al. 2006; Batra et al. 2019). Many of these (glyco) proteins belong to the defense family and are vital for membrane stabilization during immune responses mounted against the spermatozoa in the FRT (Légaré et al. 2017; Kirchoff 1998; Holland and Nixon 1998). The presence of such a peripheral coat presumably assists the spermatozoa in the FRT to survive the immunologic milieu of the FRT and ultimately fertilize the egg. The post-testicular sperm surface remodelling events continue till ejaculation as a result of which the spermatozoa acquire a substantial glycocalyx (Fig. 15.2) which performs unique reproduction-specific functions, e.g., intercellular recognition and communication.

Hence, the post-gonadal maturation of the spermatozoa is a consequence of highly intricate sequential and dynamic interactions of the spermatozoa with the unique components in the discrete luminal segments of the epididymis. The differential gene expression and protein secretion along the MRT are responsible for the
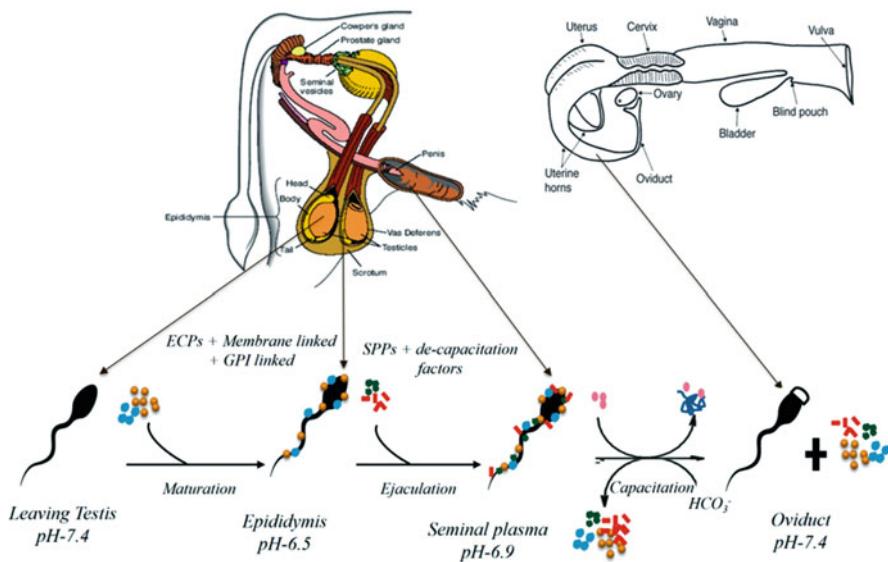
Fig. 15.1 Sperm surface remodeling events in the MRT
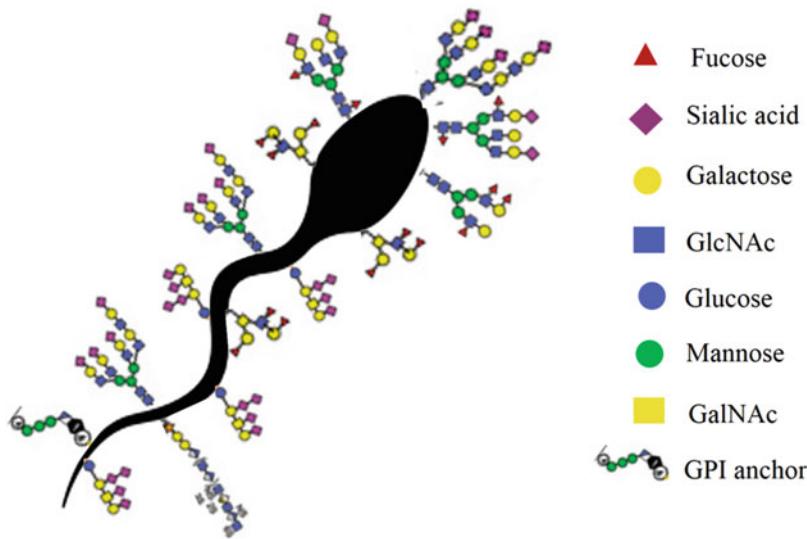

Fig. 15.2 The structural diversity and complexity of sperm glycocalyx

highly specific luminal-microenvironment in the MRT segments that interacts dynamically with the traversing spermatozoa. Simultaneous with these changes in their plasma membrane, the morphologically normal spermatozoa attain not only the motility, but also the competence for ligand recognition on the zona pellucida to

fertilize the egg (Gatti et al. 2004; Cornwall 2014; Cooper 2012; Caballero et al. 2010).

15.2 Beta-Defensins: Expansion of Functional Repertoire Beyond the Microbicidal Role

The various secretagogues present in the epididymal lumen include BDs like Spag 11, Bin1b, and DEFB-126, which are applied as a maturation-antigen coat onto the surface of traversing spermatozoa (Zhou et al. 2013; Zhao et al. 2011; Hedger 2015; Pujianto et al. 2013; Tollner et al. 2012). The functional roles played by these host defense peptides (HDPs), especially the CA-BDs, in reproduction and fertility are diverse and equally complex (Dorin and Barratt 2014; Whiston et al. 2017). The multifunctional roles of the BDs in male fertility have gained substantial attention since the pioneer report by Li et al. regarding the function of a Bin1b (an epididymal secreted BD) in reproduction. Bin1b was demonstrated to be maximally expressed in the caput epididymis assisting in sperm maturation and their storage in the epididymis before ejaculation. Moreover, it also exhibits the characteristic antimicrobial activity of BDs, thereby inhibiting the microbial invasion in the testes (Li et al. 2001). Many BDs are now known to interact with the spermatozoa during their voyage in the epididymis, which presumably act chronologically and synergistically helping them attain maturity, immune-evasion, and fertilizing abilities (Robaire and Hinton 2015; Sullivan and Mieusset 2016; Belleannée et al. 2012). These BDs are expected to localize to various regions of the spermatozoa depending on their functional roles. We have recently demonstrated that different defensins indeed vary in their spatial distribution patterns (Batra et al. 2021). For example, the buffalo beta-defensin 129 (BuBD-129) was found to span the entire buffalo sperm, while the BuBD-126 covered the acrosomal region and the tail of buffalo spermatozoa (Fig. 15.3).

Interestingly, the CA-BDs have been demonstrated to maintain the spermatozoa in the non-capacitated state before they reach the ampullary-isthmus junction in the oviduct, thus preventing premature capacitation (Colledge 2013). Further, they also
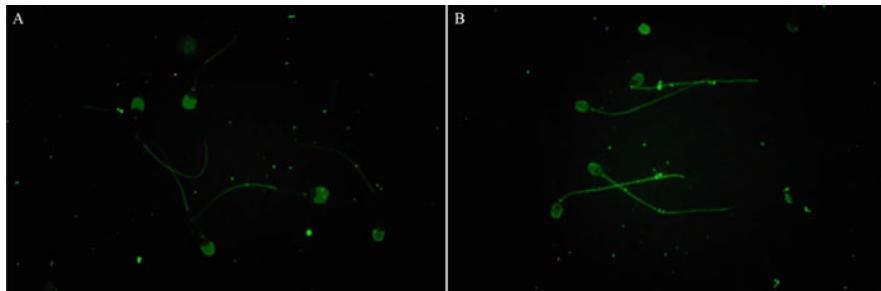

Fig. 15.3 Differential spatial distribution patterns of BuBD-126 (A) and BuBD-129 (B) on the buffalo sperm surface

assist the spermatozoa to overcome the physical barriers in the FRT such as cervical mucus (Tollner et al. 2008) and to thwart the immune responses mounted in the uterus and other segments of the FRT (Yudin et al. 2005b). Besides, they have also been implicated in the acquisition of sperm motility in the epididymis before capacitation (Fernandez-Fuertes et al. 2016) and for the formation of the oviductal sperm reservoir (Lyons et al. 2018). The miscellany observed in the functional repertoire of BDs is believed to be the result of recurrent duplications which over time have led to enough sequence and functional divergence (Batra et al. 2019). The high rates of random genetic drift coupled with diversifying selection pressure acting on these genes have widened the spectrum of their functional activity. The duplication events appear to initiate a period of relaxed selection, where one of the duplicates maintains the wild-type gene function, while the other duplicate is free to explore a new functional space (Meade and O'Farrelly 2018; Narciandi et al. 2011). It has recently been demonstrated that the CA-BDs appear to have evolved under an intricate balance of contrasting selective pressures and restraints expanding the suite as per the niche inhabited by a species (Batra et al. 2019). The number of BDs identified in a species has been reported to vary vis-à-vis its microbial load, historical contingency, genetic drift, and disparate ecological niches occupied (Meade and O'Farrelly 2018). Expectedly, our sperm surface proteomics study revealed the presence of several beta-defensins on the buffalo sperm surface including the 2 CA-BDs (BuBD-129 and 126) which appear to interact with the traversing spermatozoa surface (Batra et al. 2021).

15.3   Preferential Secretion of the Beta-Defensins in the MRT

Until now, attention has been given mainly to the antimicrobial functions of the BDs in the digestive and respiratory tracts, vis-à-vis their functional roles in the MRT. Nonetheless, the BDs secreted in the lumen of the epididymis are now known to be dynamically involved in the maturation of spermatozoa, their survival, and performance in the FRT (Zhao et al. 2011; Semple and Dorin 2012; Légaré et al. 2017; Girouard et al. 2011; Semple et al. 2015; Meade et al. 2014). The BDs along with other sperm surface proteins are either GPI-linked or adsorbed through various non-covalent interactions on the surface of the traversing sperm in the epididymis (Holt 1980). Many reports have emerged about the preferential regulation of BD gene expression in the MRT in a Spatio-temporal manner (Colledge 2013; Ribeiro et al. 2017; Batra et al. 2019; Belleannée et al. 2012; Hu et al. 2014). The functions of the epididymal BDs have been correlated with reproduction physiology and it is generally believed that they reinforce the activity of each other (Ribeiro et al. 2016; Augustin et al. 2011; Navid et al. 2012). Such BDs often include the products of a specific BD cluster, e.g., hBD-126 from the BD cluster on human chromosome 20 and DEFB-126 from the BD cluster on macaque chromosome 10. The products of these specific BD clusters have been addressed as class-A beta-defensins (CA-BDs) to distinguish them from other BDs which are mainly microbicidal in function. The transcripts of the reproduction-specific BD cluster have been found to express in the
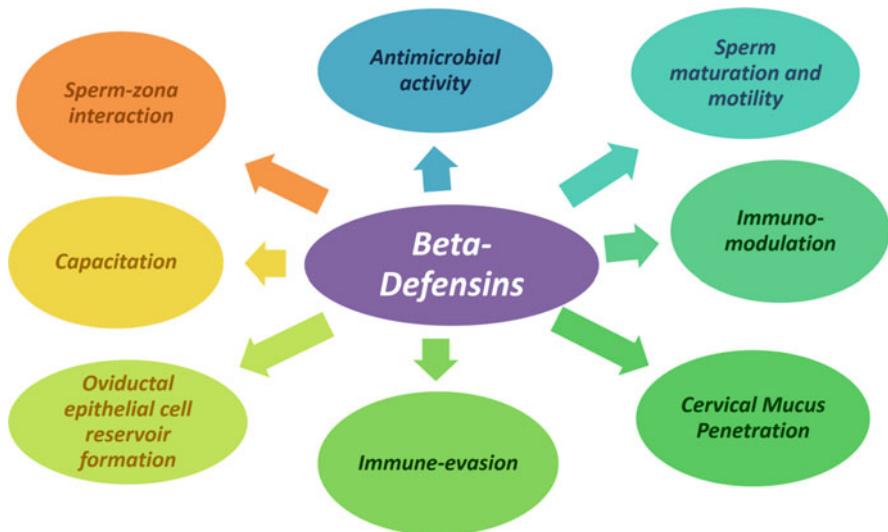
Fig. 15.4 The functional diversity of beta-defensins

epididymal epithelia and their finished protein products are secreted into the epididymal lumen and ultimately applied onto the sperm surface (Hall et al. 2002, 2007; Dorin and Barratt 2014; Yu et al. 2013; www.mrgd.cgi). The CA-BDs have been observed to possess extended tail lengths, high levels of glycosylation, and preferential secretion in the MRT and are majorly involved in male reproductive functions (Narciandi et al. 2011). For example, the DEFB126 (also known as ESP13.2), which has drawn much attention lately, is known to be adsorbed onto the surface of the macaque spermatozoa during SSR events in the epididymis (Tollner et al. 2011, 2012). Conversely, we have observed the expression of CA-BD genes in nonreproductive tissues of buffalo which indicates their active antimicrobial roles in these somatic tissues (Batra et al. 2019). Overall, it is generally accepted that the adsorption of these proteins in the epididymis moulds the sperm surface preparing it to fertilize since the testicular sperm are incompetent to do so. Molecules with such disparate and critical roles in reproduction (Fig. 15.4) deserve to be further explored and characterized for unveiling the complete diversity of their pleiotropic functions.

15.4 Regulation of the Male-Specific BDs

The BDs are regulated primarily by the androgens; nevertheless, an intricate regulation of BD gene expression has also been reported in multiple species (Ribeiro et al. 2015; Robaire and Hinton 2015; Ribeiro et al. 2017). The disparity in androgenic regulation of the BDs presumably depends on the epididymal cell type and the regions of the epididymis analyzed. The various types of epididymal cells, e.g., the principal, clear, basal, and halo cells, can cross talk to provide an appropriate

microenvironment of sperm maturation (Shum et al. 2011). The spatial variation in BD gene expression and protein secretion has been implicated as one of the factors responsible for the provision of a particular microenvironment in the epididymal lumen. The idea of androgenic modulation of these genes has been underpinned by the identification of response elements for the androgen receptor (ARE) upstream of the BD genes of the reproduction-specific cluster (Barry-Reidy 2017). For example, the rat Defb15, a rodent-specific gene, is regulated by androgen as evidenced by a decline in mRNA levels following castration which could be rescued by androgen supplementation (Zhao et al. 2011). Using ChIP/PCR and RT-qPCR assays, the binding of androgen receptor (AR) to the identified androgen response element (ARE) present in the BD genes has also been confirmed by Hu and coworkers (Hu et al. 2014).

Several murine β-defensins expressed from syntenic cluster D have been demonstrated to be fully or partially responsive to androgen in the epididymis (Hu et al. 2014). The partial response, e.g., the inability to fully maintain the basal expression levels of BDs like Spag11a (Bin1b) and Spag11c in the caput epididymis of castrated rats despite testosterone treatment, indicates the involvement of other lumicrine and paracrine factors in addition to androgens (Ribeiro et al. 2015; Pujianto et al. 2013; Reyes-Moreno et al. 2008; Belleannée et al. 2012; Sullivan et al. 2011). In accordance, diverse sites for different transcription factors (TFs) have also been identified in the regulatory sequences of the BD genes, presumably subjecting them to different regulatory controls (Alva-Murillo et al. 2012; Barry-Reidy 2017; Voss et al. 2006; Meade and O'Farrelly 2018). The proximal promoter regions of many BD genes have been found to contain binding sites for nuclear TFs such as NF-κB and NF-IL6. These binding sites are deemed crucial for BD gene induction to serve an antimicrobial role upon stimulation by lipopolysaccharides (Diamond et al. 1991, 2000; Navid et al. 2012). Regulation of bovine BDs from reproduction-specific clusters has not yet been elucidated in detail. However, some of these genes are likely to be regulated by androgen in the MRT since the CA-BD expression has been reported to be absent in sexually immature males (Narciandi et al. 2011). Moreover, the temporal variation in murine gene expression in the Wolffian duct (WD) and the postnatal epididymis further complicates the understanding of their modulation (Ribeiro et al. 2016; Murashima et al. 2015; Welsh et al. 2006, 2007). The TFs mediate the gene regulation at the transcriptional level by binding to both gene-proximal and distal non-coding DNA in a context-dependent fashion. Additionally, an understanding of how the non-coding genome controls the expression of β-defensin genes would help elucidate further details of the regulation of BD gene expression, the details of which largely remain obscure. Largely, the expression of many BDs appears to be context-dependent, linked to differentiation status, cell type, and state of infection/disease.

15.5   Role in Sperm Maturation

The BDs are known to be an important component of the epididymal-secreted secretagogues and many BDs are added to the surface of the spermatozoa before ejaculation. Zhao et al. (2011), for the first time, established the role of an epididymis-specific AMP, the Defb15 in the acquisition of sperm motility and embryonic development in addition to confirming its androgen-dependent expression pattern. The immunolocalization studies found the Defb15 to be restricted in the acrosomal region of the spermatozoa isolated from the caput epididymis and the incubation of caput-spermatozoa with Defb15 antibody resulted in a significant attenuation of sperm motility. Furthermore, the total and progressive motility of the caput-spermatozoa markedly diminished when the Defb15 was downregulated by in vivo RNAi and this led to embryonic development failure. The dose-dependent microbicidal activity was also demonstrated to indicate a dual role in reproduction and pathogen defense in the rat epididymis (Zhao et al. 2011). Another BD, Spag11 has been associated with sperm motility traits in cattle, thus regulating fertility (Liu et al. 2011), whereas the β-defensin 1 deficient have been demonstrated to underlie male infertility associated with poor sperm motility in humans (Diao et al. 2014). Recently, Khayamabed et al. have demonstrated the effect of incubating the processed spermatozoa with recombinant β-defensin 1 protein (rBD-1). It was found that the rBD-1 significantly maintained the sperm motility and viability percentage vis-à-vis the processed control samples demonstrating the role of this novel BD in sperm maturation (Khayamabed et al. 2019). Furthermore, the cattle ortholog of the primate DEFB-126, the cauda epididymis-specific recombinant BBD-126 has been demonstrated to promote the motility of the spermatozoa, although the incubation was accompanied by a reduction in the ability to fertilize in vitro matured bovine oocytes (Fernandez-Fuertes et al. 2016). More recently, Aram et al. found that the percentage of DEFB-126-positive sperm in human semen is associated with normal morphology and sperm motility. It was observed that DEFB-126-positive spermatozoa were abundant in the fertile men in comparison with the infertile men with semen deficiencies supporting the notion that DEFB-126 is indispensable to sperm maturation (Aram et al. 2020). Very few reports exist indicating the role of BDs in sperm capacitation. The pioneer report by Yudin et al. demonstrated that the DEFB126 was evenly distributed along the periphery of mature macaque sperm after ejaculation. However, it was found to be released from the sperm head at the time of capacitation or after induction with physiologic activators of capacitation (dbcAMP with caffeine) which rather remove it from the whole sperm surface (Yudin et al. 2003). The removal of DEFB-126 from the head of macaque sperm was found to be concurrent with the completion of the capacitation process, which leads to the sperm-zona binding. A clear role for DEFB126 (ESP13.2) in the capacitation process of the macaque sperm by modulating the sperm surface-receptor presentation at the time of fertilization has now been established. (Tollner et al. 2004, 2012). Besides, it has been reported that homozygous deletion of a cluster of nine murine defensin genes resulted in profound male sterility (Zhou et al. 2013). The spermatozoa isolated from the caudal region of the

epididymis from the homozygous mutants underwent premature capacitation and increased spontaneous acrosome reaction which compromised the ability to bind zona pellucida. Consistent with these observations, such spermatozoa had elevated intracellular calcium levels and disrupted the microtubular structure of the axoneme (Zhou et al. 2013). Furthermore, it has recently been suggested that exogenous supplementation of human beta-defensin 1 (hBD-1) significantly improved sperm motility and influenced sperm quality in human subjects (Zupin et al. 2019). More recently, deficiency of beta-defensin 108 has been implicated in decreased sperm motility in blue fox, Vulpes lagopus (Wu et al. 2021). The numerous reports linking BDs and male fertility suggest that these HDPs are essential in the creation of a mature male gamete capable of survival and fertilization in the FRT. The discussed reports indicate that within the FRT, the BDs may or may not play a role in innate immunity; however, they are involved in the sperm capacitation/fertilization process. Further investigation of the BDs will be important for understanding the causes of infertility and research on BDs is warranted to elucidate the mechanisms of acquisition of fertilizing abilities and infertility in the mammalian spermatozoa.

15.6 Role as an Immune-Protective Shield of the Spermatozoa

As mentioned earlier, the ability to distinguish the 'self' from the 'non-self' antigens is a crucial property of the immune system (Medzhitov and Janeway Jr. 2002; Zasloff 2002; Transforming glycoscience: 2012; Varki 2011). The spermatozoa must acquire coats that hide the unique and allogenic testicular as well as the epididymal antigens that are considered foreign by the female immune system. The BDs on the sperm surface have been proposed as immuno-protectants assisting the spermatozoa in evading the immune responses mounted against them in the FRT (Tollner et al. 2004). It was suggested by the authors that the peripheral coat comprising majorly of the DEFB126 acts as a Klingon cloak making the spermatozoa invisible to the female immune system. Later, Yudin et al. from the same group demonstrated that due to high levels of O-glycosylation in this molecule, the associated sialic moieties of the glycocalyx confer a high negative charge to the sperm surface, which appears to be responsible for their immune-surveillance evasion in the FRT (Yudin et al. 2005a). Interestingly, the idea that sperm surface-associated glycans, e.g., sialic acids, hide the inherent sperm antigens and thus assist in the immune protection of the spermatozoa was, however, proposed decades ago and recent work has underpinned the idea (Toshimori et al. 1988; Czuppon 1984; Nagdas et al. 2014; Fernandez-Fuertes et al. 2018). The experiments by Yudin et al. demonstrated that when rabbits were immunized with whole sperm, the antiserum showed a remarkably strong reaction to a single 34–36 kDa protein, which was revealed to be the DEFB126. As mentioned earlier, capacitation leads to removal of this BD from the sperm head, and when the capacitated sperm were immunized, there was a dramatic increase in the immune recognition to a variety of protein bands like PH20 (hyaluronidase), Zona pellucida binding receptors, ADAM30, and SPAM1. Such inherent GPI-linked proteins of the primate spermatozoa underlying

the cloak of DEFB-126 are rendered accessible only after the removal of the cloak defensin, DEFB-126. The role of O-linked glycosylation was established by treating the macaque spermatozoa with sialidase and O-glycanase which led to ineffective shielding of the spermatozoa from immune recognition (Yudin et al. 2005a, b). Interestingly, it was also revealed that when the fixed washed macaque spermatozoa were immunized in female macaques, the anti-DEFB126 antibody was not formed until three booster doses were injected (Yudin et al. 2005b). This finding suggested that even though DEFB126 is a male-specific glycoprotein, it goes concealed in the homologous females. Subsequently, it was confirmed that spermatozoa coated with the DEFB126 were not recognized by the raised anti-sperm antibodies because of the high level of sialic acids linked to O-glycosylated DEFB-126 protein, making the sperm invisible to immune-surveillance in the FRT (Tollner et al. 2012). The formation of antibodies against sperm surface proteins have been documented as a foremost cause of immunological infertility in humans (Bohring et al. 2001; Archana et al. 2019) and these anti-sperm antibodies directed toward the exposed sperm antigens would expectedly disrupt the fertilization process (Bronson 1999; Hataska 2000; Lotti et al. 2018; Marconi and Weidner 2017). The independent glycans and those associated with various glycoconjugates appear to hide the inherent sperm proteins which are deemed antigenic by the female immune system. Considered as the most intricate and ubiquitous modification that occurs posttranslationally (PTM), glycosylation is usually found on the surface of secreted and cell surface proteins on their serine and threonine amino acids. These residues presumably organize into clusters forming a large, negative hydration shell which is believed to protect the spermatozoa from its immediate immunologic milieu in the FRT, as in other somatic cells (Jentoft 1990; Tollner et al. 2012; Gupta et al. 2020; Diekman 2003). The glycocalyx associated with the highly glycosylated sperm surface BDs like defb22 in mice, DEFB-126 in macaques, hBD-126 in humans, and BuBD-129 in buffalo may be analogous to other glycocalyx extracellular coats which are known to protect the cell (Yudin et al. 2008; Schauer 2004; Schroter et al. 1999; Batra et al. 2020; Tollner et al. 2011, 2012). We have recently demonstrated that the differential glycomic endowment on spermatozoa from high and low fertile bulls confers a selective advantage to high fertile bull spermatozoa from recognition, phagocytosis, and NETosis by female neutrophil cells (Batra et al. 2020). We addressed the 3-D spatial configuration of the monosaccharide units of the glycocalyx as Sperm-Associated Glycan Topography (SpAGT). The observed difference in the sperm phagocytosis and NETosis was attributed to the lower abundance of glycans in LF-SpAGT in comparison to the HF- SpAGT. The lower abundance of O-linked glycans apparently transduces a varied recognition signal presumably to the bound pattern recognition receptors (PRRs) of the interacting neutrophils, resulting in elevated rates of recognition, phagocytosis, and NETosis of LF-spermatozoa by female neutrophil cells (Fig. 15.5). Thus, commensurate with the "Glyco-evasion hypothesis" (Kreisman and Cobb 2012), a higher abundance of glycans on HF bull sperm modulates the host (female) immune response resulting in immune-evasion of such spermatozoa (Batra et al. 2020). Besides, the cell surface glycocalyx is also thought to protect the sperm from enzymatic attack (Cornwall 2014) and accordingly
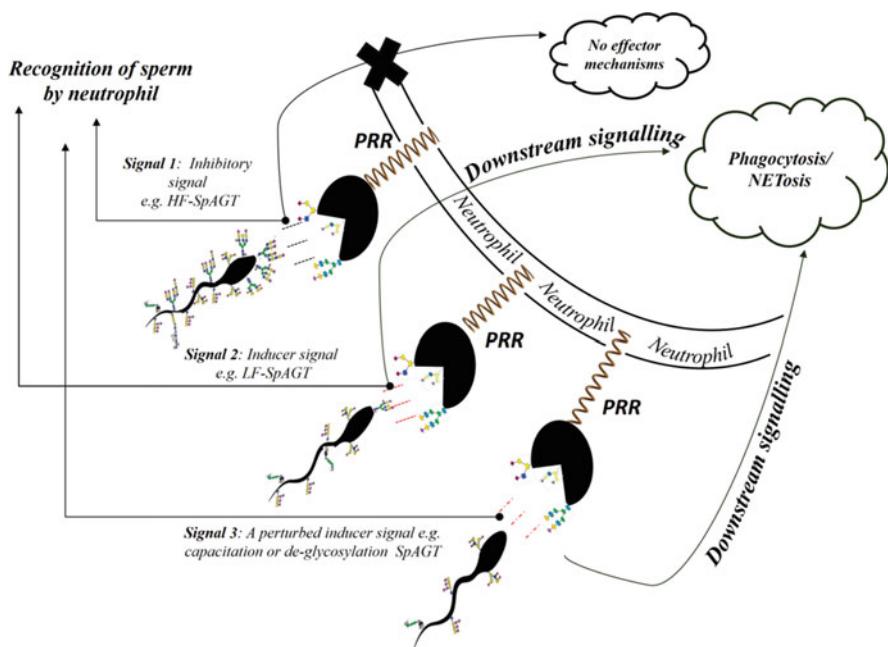
Fig. 15.5 The differential immune response to HF or LF-SpAGT by PRRs of neutrophil cells

the defb22 in mice was shown to be refractory to protease activity (Jones and Brown 1987). Therefore, the epididymal secretions have been anticipated to set up a shielding barrier against the enzyme activity of various body fluids in the MRT as well as in the FRT (Kelly 1995; Hinton et al. 1996; Archana et al. 2019; Voisin et al. 2019).

It is now widely accepted that the allogenic testicular and epididymal antigens are shielded by the peripheral coat of BD-associated glycans which is considered crucial for sperm survival in the FRT. The BDs are now deemed essential not only for sperm maturation in the male, but also for protecting the mammalian spermatozoa from the immune attack in the FRT (Archana et al. 2019). It has, therefore, been proposed that such BD cloaks could be one of the major mechanisms for sperm protection in the primate FRT (Tollner et al. 2012; Dorin and Barratt 2014).

15.7   Role in Cervical Mucus Penetration

Considered among the strongest markers of fertility, the penetration of a visco-elastic material like cervical mucus or hyaluronan gels is an indicator of the fitness of the spermatozoa (Mortimer et al. 2013). It was proposed twenty years ago that capacitation of the spermatozoa leads to the loss of that component of sperm surface which is essential for transport through the cervical mucus (Katz 1991). Even so, the sperm surface molecule that imparts this function to the spermatozoa couldn't be

identified, until recently. As evident from the several independent observations, the female tract doesn't just act as a simple conduit for sperm passage rather, it selects for the most competent spermatozoa and preserves its viability (Eisenbach 2003; Eisenbach and Giojalas 2006; Tecle and Gagneux 2015; Bianchi and Wright 2016). The first physical barrier that the spermatozoa need to surmount is the cervix where major selection and limiting of spermatozoa numbers occurs. It is believed that the selection process happens chiefly due to the interactions of the spermatozoa with the negatively charged mucins of the cervical mucus (CM). The insemination in the vagina by ruminants and primates thus facilitates the selection of competent spermatozoa by the CM (Alghamdi et al. 2009, 2015). For example, it has been established in humans that the majority of the spermatozoa leak out of the vagina after within 30 minutes of coitus (Baker and Bellis 1993) and the remaining fraction is seemingly drawn into the cervix, at least initially, by the contractions of the uterus during coitus (Drobnis and Overstreet 1992). Later, for the ensuing movement in the cervix and the upper FRT, the sperm must propel themselves via vigorous flagellar movements (Katz 1991). In addition, the surface properties of the spermatozoa should be tailored to allow efficient and unhindered movement in the CM. It is now known that the DEFB-126 coat of macaque spermatozoa, which is the most peripheral component of macaque sperm, remains attached to the sperm surface during and after its penetration of CM (Yudin et al. 2005a; Tollner et al. 2012). Whether this coat of BD assists in cervical mucus penetration (CMP) was first revealed by Tollner et al. in 2008 based on certain established facts in macaques. It had previously been established in macaques that the CM consists of negatively charged mucin molecules (Cone et al. 1998; Lagow et al. 1999). The DEFB126 which cloaks the sperm provides a negative charge to sperm surface (Yudin et al. 2005b) which was demonstrated to facilitate sperm penetration of cervical mucus (Tollner et al. 2008a). The negative charge assists in transmigration through the negatively charged mucin molecules through the Columbic interactions that play a decisive role in this process (Tollner et al. 2011). It is now recognized that the sialic acid moieties, e.g., existing on the O-linked DEFB-126, are crucial for sperm infiltration and unabated movement in the cervical mucus (Tollner et al. 2012; Fernandez-Fuertes et al. 2018). This is because of the negative charge on these moieties that confer biological activity to DEFB126 which thus forms a negative charge shell over the entire sperm surface. Any perturbations to this negative charge should interfere with the CMP. The cationic polymers, e.g., PLP (Poly-L-lysine), are well-known to neutralize the negative charge on the cell surface resulting in changes in cellular functioning (Shier et al. 1984; Pugliese et al. 1989; Steadman et al. 1990). The addition of PLP to macaque sperm was found to coat the entire sperm surface, its distribution corresponding to the presence of sialic acid on the sperm surface, which significantly blocked the CMP by macaque spermatozoa (Yudin et al. 2005a). These sialic acid molecules are added to the sperm surface during spermatogenesis, epididymal maturation, and by incorporation of sialylated seminal fluid components during ejaculation (Ma et al. 2016). It has also been demonstrated that the treatment of sperm with NMase (neuraminidase) led to abrogation of CMP ability at par with inhibition of CMP attained with ACT (activators of capacitation) treatment that is

believed to remove DEFB-126 from the macaque sperm surface. Interestingly, soluble DEFB126 added to such de-glycosylated spermatozoa restored the CMP ability (Tollner et al. 2008). The treatment of cattle sperm with NMase was found to lead to decreased CMP ability in a time-dependent manner, as well as a decrease in overall and progressive motility has been observed (Fernandez-Fuertes et al. 2018). This indicates that a negative charge on the surface of spermatozoa, e.g., associated with DEFB-126, is required for their unabated movement past the negatively charged oligosaccharides of the mucin molecules in cervical mucus (Cone et al. 1998; Lagow et al. 1999; Tollner et al. 2012). The application of negatively charged glycans in the form of peripheral coats appears to be a conserved feature of sperm maturation in many mammalian species (Bedford 1963). It is now well-established that the spermatozoa maintain the negative charge until completion of the sperm capacitation in the oviduct because ACT treatment impeded the CMP by macaque spermatozoa, as mentioned earlier. The importance of negatively charged sialic acid moieties in CMP is highlighted by the fact that men carrying a mutation in the DEFB126 gene are subfertile due apparently to a reduced ability of their sperm to penetrate cervical mucus (Tollner et al. 2011). This DEFB-126 variant with a 2 nucleotide deletion, which shifts the reading frame, produces a non-stop mRNA and is deficient in glycosylation, thereby possessing perturbed sperm-associated glycans topography. As expected, such sperm had markedly impaired mobility in the hyaluronan gels which are the surrogate for peri-ovulatory cervical mucus. This del/del mutant allele was surprisingly very common in human population cohorts across the globe, despite translating to a 30% reduction in the rate of live births (Tollner et al. 2011). More surprisingly, such spermatozoa had normal functional parameters considered conventionally, e.g., like shape, motility, and sperm count. Either the allele confers a heterozygous advantage or this can be a case of antagonistic pleiotropy wherein one or more traits are beneficial to fitness but others are detrimental (decreased fertility). The enigma is yet to be resolved which prima facie appears to be a putative compromise between utilitarian selection and sexual selection.

15.8 Role in the Formation of Oviductal Sperm Reservoir

The site of semen deposition and the number of spermatozoa (millions to billions) vary widely across species (Yanagimachi et al. 1994; Alghamdi et al. 2009; Alghamdi et al. 2015). Only a meagre fraction of the spermatozoa, however, successfully migrates to the site of fertilization and it appears that the ratio of male gametes and female games approaches unity (Birkhead 1998; Armon and Eisenbach 2011; Tollner et al. 2008b; Coy et al. 2012). Such intricate organization of the ratio of gametes is attained by a sustained release of capacitated and mature spermatozoa from the oviductal epithelial cells (OECs) in response to the ovulation signals (Hunter 2008, 2012; Miller 2015; Holt and Fazeli 2010). The gradual release of sperm is possible because of the fact that the capacitation isn't synchronized in vivo, i.e., all sperm do not undergo capacitation at the same time, those who do, detach

from the OECs due to the loss in sperm surface components that had an affinity for the OECs. This loss helps the release of sperm from the reservoir (Suarez and Pacey 2006; Holt and Fazeli 2010; Suarez 2016). The spermatozoa selectively bind to the oviductal epithelial cells (OECs) which appear to form the isthmic sperm reservoir (Holt and Fazeli 2010). This reservoir plays a significant role in successful fertilization (Rodríguez-Martínez et al. 2005; Bianchi and Wright 2016; Ikawa et al. 2010) because sperm binding has been shown to select a population of competent spermatozoa (Teijeiro et al. 2011; Coy et al. 2012; Holt and Fazeli 2010) and regulate sperm function (Miller 2015). It has been demonstrated that in macaques the DEFB-126 plays an integral role in the in vitro sperm binding to the OEC explants. The terminal glycans on DEFB126 appeared to assist sperm-OEC binding and the in silico analysis for potential glycosylation sites predicts ten potential sites on DEFB-126 (Yudin et al. 2003; Tollner et al. 2012). It was demonstrated that the NMase (neuraminidase) treatment of DEFB126 inhibits the binding of non-capacitated spermatozoa to the OECs, as the treatment with anti-DEFB-126 antibodies. Moreover, the capacitation-associated loss of DEFB126 from the head and mid-piece led to a drastic attenuation in the in vitro binding ability of the spermatozoa to bind the OEC explants obtained from the follicular phase oviducts of the female macaques. A preparation of DEFB126 given in the form of "add-back" to capacitated sperm completely reinstated the spermatozoa's binding ability comparable to that of the non-capacitated sperm (Tollner et al. 2008b). Recently, it has been demonstrated in cattle that the recombinant beta-defensin 126, rBBD-126, significantly increased the ability of the ejaculated spermatozoa to bind the bovine oviductal epithelial cell (BOEC) explants (Lyons et al. 2018). The anti-BBD126 antibody was found to block the increase in sperm binding capacity mediated by BBD126. In conclusion, several lines of evidence suggest that BDs like BD-126 assist the binding of sperm to OECs during the follicular phase of the reproductive cycle in disparate mammalian species.

15.9 Structural Similarity with Immunomodulators and Immune-Regulatory Roles

The BDs are known to link the innate and the adaptive immune systems by initiating cross talk between these two arms of immunity (Shum et al. 2011; Donnarumma et al. 2016). Evidence is rapidly accumulating about the role of BDs in the modulation of host immune responses during inflammation, infection, and other non-immunological physiologic events. They induce an adaptive immune response by mobilization of the immune-competent cells through chemotaxis permitting the host to curb, delay, or avoid the pathogenesis (Yang et al. 2007; Jiang et al. 2018; Yang et al. 2018). They are capable of modulating the immune response towards either the pro-inflammatory Th1 or anti-inflammatory Th2 responses by modulating downstream signaling cascades of the chemokine receptors or CCRs (Funderburg et al. 2011; Semple et al. 2010; Fusco et al. 2017; Feng et al. 2017). The expression of BD genes can either be constitutive or inducible (Otri et al. 2012; Semlali et al.
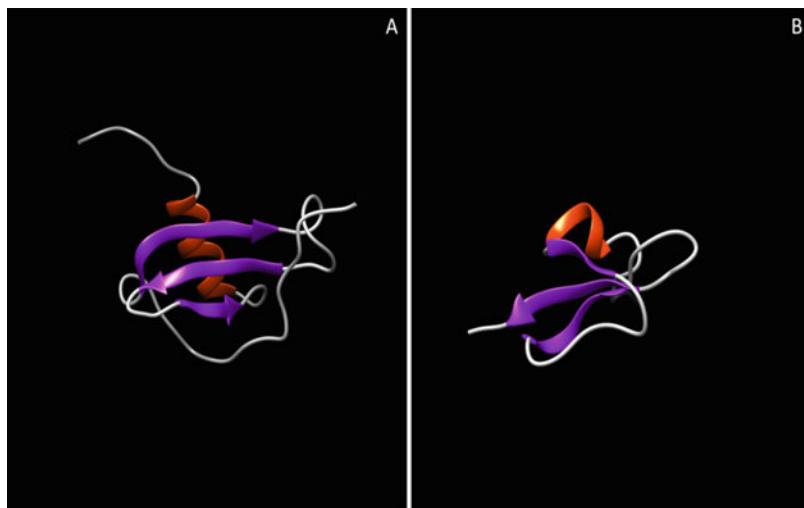
Fig. 15.6 The similarity between the tertiary structures of the CCR ligand chemokine CCL20 (**a**) and hBD-2 (**b**)

2012; Lüthje et al. 2014) and their regulation can either be positive or negative (Pujianto et al. 2013; Rengaraj et al. 2018). BDs contribute to innate immunity not only by killing the pathogens after invasion, but also by immune regulation. In accordance, many BDs possess positive immune-modulatory properties, whereas others have negative modulatory roles (Sorensen et al. 2005; Semple and Dorin 2012). Their constitutive expression has been reported irrespective of the presence of pro-inflammatory factors, e.g., LPS, air pollutants, or asbestos (Goldammer et al. 2004; Bagnicka et al. 2010; Tu et al. 2015; Donnarumma et al. 2016). Alternatively, they have also been found to be upregulated in response to pathogenic infection (Whelehan et al. 2011; Tu et al. 2015; Fruitwala et al. 2019). These innate effectors (BDs) can interact with the adaptive immune system by interacting with Toll-like, chemokine, and other receptors present on the lymphoid and myeloid cells, thereby modulating the immune response by the host (Hedger 2015; Silva et al. 2011; Jiang et al. 2018; Yang et al. 2018). Interestingly, some BDs use the same receptors as does the chemokine CCL20, e.g., chemokine receptor type 6 CCR6, suggesting that either there is a structural similarity between these proteins or they have an analo- gous mechanism of receptor recognition and activation (Semple and Dorin 2012). The structural-activity-relationship studies have shown that the interaction of BDs with eukaryotic cells depends on its hydrophobicity, while the microbicidal activity is determined by side chains of basic residues. For example, the hBD2 and the chemokine CCL20 (MIP-3α) have been reported to have similar three-dimensional structures (Perez-Canadillas et al. 2001) explaining the reason for the ability of both to trigger the CCR6 receptor (Fig. 15.6).

Surprisingly, the chemokines and the BDs both have been demonstrated to modulate not only the motility of the spermatozoa, but also the sperm–oocyte or

sperm–reproductive tract interactions (Yu et al. 2013; Navid et al. 2012). For example, DEFB1 acts on the CCR6 present on sperm leading to the mobilization of the calcium which is deemed important for the acquisition of sperm motility. Any hindrance in the CCR6 function leads to diminished motility and also the anti-bactericidal activity of the normal sperm, which is associated with poor sperm motility and infection. This indicates the role of DEFB1 in protecting male fertility and thus suggesting the diagnostic potential of DEFB1 and CCR6 (Diao et al. 2014). Thus, from the perspective of immune regulation, BDs appear to engage several cell surface receptors potentially acting as chemokines and may promote chemotaxis. Furthermore, pieces of evidence of involvement of NF-κβ and mitogen-activated protein kinase pathways leading to AP-1 transcriptional activation as well as the epidermal growth factor receptor (EGFR) pathway for the regulation of BD expression also exist (Klotman and Chang 2006; Sorensen et al. 2005; Johnston et al. 2011; Shuyi et al. 2011; Froy 2005). Thus, it appears that the conserved defensin motif reflects involvement in cell signaling rather than antimicrobial activities. Largely, the AMPs serve not only to recruit various immune cells, but also play a role in the maturation and proper functioning of these cells.

15.10 Conclusion

Despite their promising potential in improving animal health and selective breeding programs, the research on the role of BDs in male fertility has been largely overlooked. Can these be used to increase the fertilizing ability of an LF sperm? How do these AMPs help to not only achieve but maintain male fertility? A better understanding of BDs and their correlation with sperm function will help to gain better insights into the immunology of reproduction. The lineage-specific expansion of BDs holds promise for targeted breeding and holds enormous potential for improved intervention strategies. The challenge remains to relate the primary sequence disparity with the functional implication in BDs and a concurrent effort is needed to fully understand the role of this evolutionary labile gene family in livestock immunity and reproduction. A complete elucidation of their physiological functions in the MRT holds enormous potential to achieve male fertility optimization in a farm setup.

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Epigenetic Bearing on Fertility in Farm Animals

16

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Abstract

Epigenetics studies alterations in gene expression occurring without changing the nucleotide sequence. Epigenetic modifications are mediated by DNA methylation, histone modifications, and RNA-based gene expression regulation, although influenced by environmental and nutritional factors. It has been well-established in both humans and farm animals that DNA methylation plays a crucial role in epigenetic reprogramming of germ cells, and any aberrance in this process could generate incompetent germ cells unable to fertilize. Additionally, aberrant histone modifications and protamine expression are directly related to infertility. The present chapter provides a comprehensive understanding of mechanisms of epigenetic modifications in germ cells and attempts to correlate the implications of DNA methylation during epigenetic reprogramming, epigenetic potential of the nuclear proteome, and abnormal protamine expression with the fertility in farm animals. It also highlights the epigenetic alterations associated with ART failure, ageing oocytes, and poor fertilizing ability.

Keywords

Epigenetics · Assisted reproductive techniques · Fertility

16.1 Epigenetics—One Genotype Many Phenotypes

Epigenetics refers to the changes in gene expression that occur without a change in nucleotide sequence (Morris 2001). It is a natural biological event that is influenced by nutritional, climatic, and disease factors. The differentiation of brain, liver, and

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other cellular features from the same DNA sequence in an individual is the simplest example of this phenomenon. Epigenetics manifests itself in a variety of ways, including DNA methylation, RNA-based gene regulation, and histone modifications (Dada et al. 2012). A number of epigenetic processes are being discovered, and their role in various disease conditions is slowly becoming clear. As the emerging views regarding the transfer of inheritable epigenetic processes are being unravelled, this field is being closely monitored because it has been implicated in a number of genetic disorders and diseases (Zhai et al. 2008). Furthermore, the increased accessibility to high throughput sequencing technologies has added an entirely new paradigm to the study of epigenetics. The intricacies in the epigenome data are difficult to deal with because the epigenetic features not only exhibit inter-individual variations, but also variations between dissimilar cells and tissues of the same individual (Lister et al. 2009). These distinct features are the result of the aforementioned epigenetic processes, emphasizing the complex interrelationship between genotype and phenotype.

16.2   Epigenetic Regulation of Germ Cells

Nature has provided chromatin organization which includes the DNA and histones for the orderly expression of genetic information, which is further influenced by epigenetic changes in them (Goldberg et al. 2007). The electrostatic contacts between these two oppositely charged entities (Histones being positively charged because of the presence of positively charged amino acids such as lysine and arginine, while the DNA being negatively charged) allow them to interact. These associations between histones and DNA are regulated by epigenetic mechanisms, resulting in the induction or suppression of cellular processes such as transcription depending on the needs of the cell. In addition, a compact heterochromatin is needed for chromosomes to align and recombine efficiently wherein the DNA is tightly associated with the histones. Contrarily, the transcriptional processes require euchromatin, i.e., a loose binding state between DNA and histones (Allis et al. 2007). As a result, a clear fluidity of switching epigenetic states across all of developmental regulation and cellular functioning tends to ensure that the destined events occur at intended time intervals.

16.2.1 Kinetics of DNA Methylation in Germ Cells

During gastrulation, separation of somatic and germ cells occurs (Swartz and Wessel 2015; Wu et al. 2016) and epigenetic changes at this stage take place, which are considered as one of the biggest epigenetic changes in any cell population known to date (Tang et al. 2016). The epigenome of primordial germ cells (PGCs) in human and mouse is largely erased at this stage, with an extensive wave of demethylation

and simultaneous histone modification changes (Seisenberger et al. 2012; Guo et al. 2015; Eguizábal et al. 2016). This extensive reprogramming is thought to provide a clean slate of epigenome, which is required for differentiation into a new organism in the next generation. This major wave of genome-wide demethylation is completed by the 8-cell stage in bovine embryos (Jiang et al. 2018; Duan et al. 2019a, b). During development of embryo, gametes differentiate and acquire epigenetic signatures specifically with regard to gamete sex and type, where they regain functional DNA methylation. Duan et al. (2019a) observed higher methylation in sperm (72.5%) than oocytes (~30%), and after fertilization, it decreased till 8 celled stage (15.3%) to the lowest point (Duan et al. 2019a, b), coinciding with the onset of embryonic genome activation (EGA) (Misirlioglu et al. 2006; Jiang et al. 2014). Then doubling of de novo methylation occurs at 16 celled stage as observed by whole genome bisulfite sequencing (Duan et al. 2019a, b) and immunostaining of 5methyl cytosine (de Oliveira, Dogan et al. 2013). Jiang et al. (2018) showed that bovine sperm and oocytes have several differentially methylated regions (DMRs), where DMRs specific to bovine sperm were highly enriched in long terminal repeats and they immediately lost methylation in embryo; whereas oocytes-specific DMRs were often present in exons and CpG islands (CGIs), which were gradually demethylated across cleavage states (Jiang et al. 2018). DNA methylation is most predominantly concentrated in long terminal repeats and CpG regions which are dispersed all over the genome. Methylation is usually absent in the promoter regions of the genome wherein the occurrence of CpG regions is particularly high (CpG islands). This absence of methylation accounts for gene expression owing to the accessibility of sites for the binding of transcriptional machinery (Aapola et al. 2002). Contrarily, the methylation of these promoter regions and differentially methylated regions of the imprinted genes causes gene silencing by obscuring transcription initiation sites. The DNA methyltransferases, which are divided into two categories, viz., the de novo methyltransferases (DNMT 3A and DNMT 3B) and maintenance methyltransferases (DNMT1), catalyse the process of DNA methylation (Goll and Bestor 2005). Besides, a novel methyltransferase, DNMT3L, has also been reported that has been demonstrated to promote DNMT3A but not DNMT3B (Ooi et al. 2007). Spermatogenesis, which leads to the development of mature spermatozoa, is one of the most meticulously controlled and highly coordinated processes. Several epigenetic alterations, involving transposable elements, imprinted genes, and other developmental processes, occur throughout male gametogenesis, all of which are necessary for the formation of healthy spermatozoa. These mechanisms rely heavily on DNA methylation. Transposable elements, which are silenced by DNA methylation, are the initial ones to undergo epigenetic modifications. DNA transposons, long terminal repeat retrotransposons, LINEs, and SINEs are examples of these elements, which, if not silenced, will spread throughout the genome and would dysregulate the genetic rearrangements (Deininger et al. 2003). The newly discovered DNMT3L has been demonstrated to aid in the silencing of transposable elements (Hata et al. 2006).

16.2.2 Genomic Imprinting

In bovine oocyte growth, DNMT1, DNMT3A, and DNMT3B (a cooperative homo-
logue; DNMT3L) were found to be associated with establishment of imprinted genes
(O'Doherty et al. 2012; Duan et al. 2019a). Later on, during development phases,
DNMT1 and DNMT3A are present till 8 celled stage (Golding et al. 2011; Duan
et al. 2019a), then DNMT3B seems to be majorly responsible for further DNA
methylation events (Dobbs et al. 2013; Duan et al. 2019a). Besides these DNA
methylation writers, DNA methylation erasers have been identified as ten-eleven
translocation (TET) family of proteins (DNA methylcytosine dioxygenases; TET1,
TET2, and TET3) (Bakhtari and Ross 2014; Huang et al. 2015), which are known to
oxidize 5-methyl cytosine to form 5-hydroxymethylcytosine (5hmC),
5-formylcytosine, and 5-carboxylcytosine (Ito et al. 2011; Wu and Zhang 2014;
Neri et al. 2016). TET3 and TET2 are abundantly found in bovine oocytes till
4 celled stage Duan et al. 2019a, indicating TET-mediated DNA demethylation
event which continued after fertilization (Duan et al. 2019a). However, TET1 is first
seen at 4 celled stage and its expression is maintained till 16 celled stage (Duan et al.
2019a), in concert with its known function of promoting pluripotency of inner cell
mass in blastocysts (Ito et al. 2010; Seisenberger et al. 2013). For histone methyla-
tion in bovine embryos, EHMT1/2, SUV39H1/2, SETDB1, and EZH2 are
characterized as writers for H3K9me2, H3K9me3, and H3K927me3, respectively
(McGraw et al. 2007; Ross et al. 2008; Golding et al. 2015; Zhang et al. 2016).
Enzymes responsible for histone demethylation (erasers) from H3K4 (KDM1A,
KDM1B, KDM2B, KDM5A, KDM5B, and KDM5C), H3K9 (KDM3A, KDM3B,
KDM3C, KDM4A, KDM4B, and KDM4C), and H3K27 (KDM6A, KDM6B, and
KDM7A) were identified by Glanzner et al. (2018) in Bovine (Glanzner et al. 2018).
Demethylation of H3K27me3 during cleavage in cattle is catalysed by KDM6B
(JMJD3) activity and knockdown of KDM6B in cattle oocytes resulted in decreased
demethylation of H3K27me3 leading to the impaired EGA and reduced develop-
ment of blastocysts, in both parthenogenetic (Canovas et al. 2012) and fertilized
(Chung et al. 2017) embryos. As previously stated, sex-specific changes in the
epigenome occur at the global level during gametogenesis as a wave of DNA
demethylation, succeeded by reinstatement of chromatin modifications and DNA
methylation (Holliday 1989; Ariel et al. 1991, 1994; Sassone-Corsi 2002). This adds
to the germ line's unique trait of controlling gene function from one generation of the
organism to the next. The consistency of this resetting process is critical for
preventing detrimental epigenetic modifications from being transmitted to future
generations. The germ cells from parents who failed to undergo epigenetic
reprogramming either as a result of assisted reproductive procedures (DeBaun
et al. 2003) or exposure to damaging environmental or chemical variables produce
offspring who are more susceptible to hereditary illnesses (Rhind et al. 2003). This
phenomenon of setting up of sex-specific imprint marks on genes is termed as
genomic imprinting by which some genes are expressed in a parent-of-origin-
specific manner (Trasler 2005). The imprinted genes are known to be expressed
either from the maternal (e.g., H19) or paternal side (e.g., IGF-2). Thus, epigenetic

modifications such as DNA methylation and other histone modifications are used to achieve this monoallelic gene expression. These epigenetic marks are generated in the germline and are carried by an organism's somatic cells. A number of imprinted genes influence post-natal behaviour, while others play a critical role in development and growth (Morison et al. 2005). The Imprinting control regions (ICRs) are critical sequence elements that control allele-specific gene expression at imprinted domains. A majority of ICRs is attributed to CpG islands and are several kilo bases long. These all are distinguished by allele-specific DNA methylation, usually observable on either the maternally or paternally inherited alleles (Delaval and Feil 2004). The allelic imprints acquired from oocytes or sperm are passed to the zygote during fertilization and are stably retained in the developing embryo, where they promote monoallelic expression of imprinted genes. The protein factor BORIS (brother of the regulator of imprinted sites, Klenova et al. 2002), DNA methyltransferases, DNMT3A, DNMT3B, and the closely related DNMT3L are involved in the development of paternal imprints in male germ cells (Kato et al. 2007). The loss of methylation in paternally imprinted sites has been found to be caused by the deletion of DNMT3L. Besides, the DNMT3A and DNMT3B-deficient spermatogonia exhibited differences in methylation patterns in the paternally imprinted sites (Kato et al. 2007). The enzyme DNMT1 has a role in the imprinting process as well. The loss of genomic methylation in DNMT1-deficient embryos was less than 30% of that observed in heterozygous or wild-type embryos. Additionally, perturbed methylation patterns have been discovered in both paternally and maternally imprinted areas (Li et al. 1992, 1993). The importance of appropriate paternal imprinting for progeny acquiring these alleles naturally has been established, as evidenced by several studies that found an increased prevalence of abnormal methylation in imprinted genes in infertile men with oligozoospermia (low sperm count) compared to normozoospermic men (Kobayashi et al. 2007; Boissonnas et al. 2010; Kläver et al. 2013). This fact has been affirmed by various genome-wide studies in humans and animals (Hammoud et al. 2011; Aston et al. 2012; Verma et al. 2014), raising the possibility that infertility/subfertility could be caused by a failure to establish precise imprinting marks or other methylation changes across the genome.

In humans as well as other mammalian species such as cattle, sheep, and goats, the establishment of precise methylation information has been demonstrated to be the most critical event during oocyte maturation and early embryonic development (Colosimo et al. 2009; O'Doherty et al. 2012; Tomizawa et al. 2013). DNA methylation is re-established in the mouse female germline during folliculogenesis after birth, and it is complete by the time the oocyte reaches the germinal vesicle (GV) stage. The DNA methyltransferases play a major role in this process (Tomizawa et al. 2013).

Furthermore, epigenetic alterations are essential for meiotic chromosomal packing, pairing, and recombination processes. For example, the chromosomes that lack DNMT3L are unable to produce heterochromatin and couple at the zygotene stage. The loss of methylation causes activation of genes which should ordinarily be silenced, e.g., the imprinted genes and other transposable elements, which results in meiotic disaster, as described by Bestor (Bourc'his and Bestor 2004). Till date, to

the best of our knowledge 53 imprinted genes are identified in bovine (Chen et al. 2016), out of which 34 are annotated (Duan et al. 2019a). In a genome-wide methylation study of bovine embryo, five maternally imprinted genes (neuronatin (NNAT), mesoderm-specific transcript (MEST), PLAG1 like zinc finger 1 (PLAGL1), paternally expressed 10 (PEG10), and small nuclear ribonucleoprotein polypeptide N (SNRPN)) were found to have higher DNA methylation in in vivo matured oocytes (Jiang et al. 2018). However, three paternally imprinted genes (H19, maternally expressed 3 (MEG3), and tumor-suppressing subchromosomal transferable fragment 4 (TSSC4)) were more methylated in the sperm (Jiang et al. 2018).

16.2.3 Chromatin Assembly Involving Post-Translational Modifications of Histones

Nucleosomes contain histone 2A (H2A), histone 2B (H2B), histone 3 (H3), and histone 4 (H4), which are all vulnerable to covalent changes like methylation, acetylation, ubiquitination, and phosphorylation during mitosis and meiosis. Gene activation and repression are determined by chromatin alterations. de Oliveira et al. (2013) evaluated the differences in bull fertility, histone retention, and expression of a histone variant (H3.3) and two core histones (H2B and H4), they observed differences in chromatin condensation between high fertile (HF) and low fertile (LF) groups, whereas they failed to found any significant differences in amount of H3.3, H2B, and H4 histones in HF and LF bulls (de Oliveira et al. 2013). Conversely, Tang and colleagues revealed the indispensable role of H3.3 in viability and male fertility through their experiment using transgenic mice carrying null mutations for H3.3 (Tang et al. 2015). Every one of the chemical changes of histones influences gene expression either alone or in combination. The histone methylation on arginine and lysine (K) residues in H3 and H4 can activate or repress genes (Lachner and Jenuwein 2002; Suganuma and Workman 2008). Histone methyltransferases perform monomethylation, dimethylation, and trimethylation alterations on H3K4, H3K9, or H3K27, which have a strictly controlled temporal expression and maintain appropriate spermatogenesis progression (Payne and Braun 2006; Godmann et al. 2007). Histone acetyl transferases are enzymes that acetylate histone at certain lysine residues, which is linked to gene activation (An 2007). This is especially important during male meiosis. Post-translational modifications (PTM) of H3 (H3K27ac and H3K27me3) were found to be associated with bull fertility, indicating that difference in levels of methylation and acetylation could be a reason for abnormal epigenetic status of spermatozoa which were unable to fertilize, activate the egg, and to contribute to embryo development as well (Kutchy et al. 2018). Apart from histone phosphorylation at numerous serine sites, activation is generally linked to histone phosphorylation (Rossetto et al. 2012). Ubiquitination at lysine residues, on the other hand, can either increase or suppress transcription (Verger et al. 2003).

16.2.4 Changes in Chromatin Structure by ATP Hydrolysis

The alterations in chromatin structure that make it accessible or inaccessible to transcription factors are mediated by a variety of processes, including DNA methylation and histone modifications (Narlikar et al. 2002). One of the most well-known of these is ATP hydrolysis-dependent. The SWI/SNF (switch/sucrose nonfermenter) family, the ISWI (imitation switch) family, and the MI-2 (myositis-specific autoantigen 2) family, all of which include a conserved catalytic ATPase subunit, have been identified thus far. These proteins move along DNA and change its conformation, such as by twisting, bulging, or spooling, to make it more or less accessible to transcription factors (Fan et al. 2003). Spermatogenesis-related abnormalities, e.g., those involving homologous recombination and DNA repair, have been reported when these proteins were knocked down.

16.2.5 Replacement Dynamics of Histones with Protamines in Sperm

A switch from the histone to a protamine-based DNA packaging arrangement allows paternal DNA to fit into the nucleus of sperm (Oliva and Dixon 1991; Balhorn et al. 2000; Balhorn 2007). Arginine and cysteine-rich proteins, the protamines appear to have evolved independently in different phyla due to a single frame-shift mutation in an ancestral H1 gene (Lewis et al. 2004; Ausió et al. 2007). In most species, two types of protamines (protamine 1 and protamine 2) are involved in wrapping the sperm DNA. While genes for both types exist in some species, such as bovine, porcine, and some primates, only one is expressed (Maier et al. 1990; Queralt et al. 1995). The diversifying selection favoring the creation of a nucleoprotamine-based chromatin structure emerged most likely as a result of its ability to bundle DNA ten times more efficiently than nucleo-histones. The sperm cell nucleus is transcriptionally dormant, and the great compression of its DNA during the final stages of development guarantees that the paternal genome is delivered to the egg safely. Balhorn's pioneering work in 2007 demonstrated how protamines (specifically P1), which associate with 10–11 nucleotides per monomer, can complex with and coil 50 kb of naked DNA ex vivo to form toroidal structures of 60–100 nm diameter and a thickness of 20 nm using biochemical, time lapse, and atomic force imaging techniques. The sperm nucleus gains a higher effectiveness in packing the paternal genome by piling these toroids, thus reducing the size of the sperm nucleus to a bare minimum. Since the sperm motility and function are known to be affected by head shape and size (Ausió et al. 2007), the nuclear dimensions are expected to be a significant requirement in facilitating optimal head shape and efficient paternal genome compaction. As a result, there is a tremendous evolutionary drive to replace histones with protamines. Transition proteins are considered to help chromatin adapt for protamine interaction by affecting DNA condensation (Meistrich et al. 2003). Transition proteins become a substantial chromatin component during the development of post-meiotic haploid spermatids. Transition proteins make up 90% of all chromatin basic proteins after histone removal and before protamine deposition. The

transition protein 1 and transition protein 2, the most well-studied of these proteins, account for around 55% and 40% of spermatid total nuclear proteins, respectively (Meistrich et al. 2003). Mice with mutations in transition proteins 1 and 2 are able to reproduce, however with lower fertility, implying that these proteins have overlapping functions (Yu et al. 2000; Zhao et al. 2001). Phosphorylated transition protein 2 is linked to less compacted DNA, which could make protamine entrance easier (Meetei et al. 2002). Hyperacetylation of histone H4 is thought to aid the transition to protamines (Sung and Dixon 1970). The reduced amounts of hyperacetylated H4 in sperm from low fertility bulls are related with loose chromatin structure, leading to defects in sperm molecular shape and function, according to a study in high fertile and low fertile Holstein bulls (Ugur et al. 2019). While it's unknown how H4 hyperacetylation causes this function, it's worth noting that CDY, a histone acetyltransferase protein encoded by the Y-chromosome, is testis-specific and rapidly acetylates H4 (Lahn et al. 2002).

16.3   Epigenome Dynamics and Implications in Fertility: Is DNA Methylation a Decisive Indicator of Fertility?

The phenomenon of methylation has been discovered to be crucial in the epigenetic reprogramming of germ cells, as discussed above. It is completely erased and subsequently re-established during germ cell development indicating its dynamic nature (Reik and Walter 2001). Any impediment or change to this process, whether owing to a lack of enzymes or substrates (donors of methylation groups such as methionine), could result in the generation of germ cells that are not fertile. According to recent research, these alterations/aberrations have a considerable negative impact on fertility. Human studies (Boissonnas et al. 2010; Hammoud et al. 2010; Poplinski et al. 2010; Sato et al. 2011; Kläver et al. 2013) have proven this fact beyond a shadow of a doubt. Methylation of imprinted genes has been examined in particular for its connection to spermatogenetic abnormalities. Furthermore, testicular biopsies of patients with non-obstructive azoospermia exhibited hypermethylation of the MTHFR promoter area in a study conducted by Wu et al. 2010b, indicating that epigenetic silencing of the MTHFR gene may play a role in azoospermic infertility. In this study that looked at the CpG methylation levels of various genes, it was discovered that majority of them were methylated differently in fertile and infertile people (Wu et al. 2010b). Furthermore, the pattern of infertility has been discovered to be linked to particular epigenetic changes, e.g., methylation anomalies in the MEST gene in oligozoospermic patients, and aberrant protamine cases were affected at KCNQ1 and SNRPN genes (Hammoud et al. 2010). Comparison of methylation in high motile (HM) and low motile (LM) sperm populations of *Bos taurus* showed variable methylation of CpG islands (CGIs) and Bovine satellite (BTSAT4) regions, whereas BTSAT4 region was found hypomethylated in HM sperm populations (Capra et al. 2019). This study suggested that methylation variation was associated with genes involved in chromatin remodeling and repetitive

element in pericentric regions, indicating importance of chromosome structure maintenance through epigenetic regulation is crucial for correct sperm functionality.

Liu et al. (2010) studied the Jmjd1 gene, which is expressed during spermatogenesis and promotes demethylation of mono- and di-methylated histone H3 lysine 9 (H3K9me1 and H3K9me2), but not trimethylated histone H3K9 (H3K9me3) (Liu et al. 2010). Expectedly, Jmjd1a knock-out germ cells had lower levels of histone acetylation. These results suggest that the Jmjd1a thus induces transcriptional activation by reducing the histone methylation and raising histone acetylation. In accord, the loss of Jmjd1a in male mice resulted in severe oligozoospermia, small testes, and infertility caused by widespread germ cell apoptosis and impeded spermatid elongation (Liu et al. 2010). There is no similar report for Jmjd1a in farm animals, making it an impossible candidate for screening bulls with poor reproductive performance. In addition, other gametogenesis regulators, such as DAZ genes, have been linked to infertility. Methylation has been shown to influence DAZ genes and homologues, which encode germ cell-specific RNA binding proteins involved in transcription, translation, and other developmental processes. They are only expressed in germ cells once differentiation genes have been demethylated, and they are essential for the formation of a distinct germ line image. Because of their importance, they've been looked into as a possible cause of spermatogenetic abnormalities. In spermatozoa of normozoospermic and oligoasthenozoospermic patients, DNA methylation patterns of promoter CpG islands of two germ line regulator genes – DAZL and DAZ, revealed increased methylation defects in the DAZL promoter of OAT (oligoasthenozoospermia) cases when compared to NZ (normozoospermia) cases (Navarro-Costa et al. 2010). Wu et al. (2016) examined the methylation pattern of the DAZ gene in infertile patients (azoospermic) and, nevertheless, reported that the methylation of the DAZ gene promoter was unrelated to male infertility (Wu et al. 2010a). Such disparate findings suggest that methylation as a predictor of infertility still needs to be supported by more substantial evidence. Non-homogeneity between samples could be the cause of such variances. Furthermore, Kläver et al. (2013) found MEST DNA methylation to be substantially linked with oligozoospermia and proposed it as a potential marker for such cases (Kläver et al. 2013).

A number of other studies have used mouse models (O'Bryan and De Kretser 2006), candidate gene sequencing (Miyamoto et al. 2003; Aoki et al. 2005; Aoki et al. 2006a, b; Hammoud et al. 2007; Hammoud et al. 2009a, b), and genome-wide association studies in humans (Aston and Carrell 2009) as well as animals (Verma et al. 2014) to explore possible genetic and epigenetic causes (methylation) of male infertility. The studies rule out the single gene polymorphisms as the cause of most occurrences of male infertility. Verma et al. (2014) found that genes related to spermatogenesis, capacitation, germ cell development, and embryonic development were differentially methylated between fertile and sub-fertile buffalo bulls, based on genome-wide profiling of sperm DNA methylation in fertile and sub-fertile buffalo bulls (Verma et al. 2014). The authors concluded that DNA methylation in spermatozoa could be an important predictor of breeding bull fertility. Besides, the allele-specific DNA methylation variations at regulatory regions of genes implicated

in piRNA control were found to be connected to impaired spermatogenesis in a microarray-based investigation (Friemel et al. 2014). Contrarily, a study by Jena et al. (2014) found that the methylation state of H19-IGF2 DMR (differentially methylated region) was not different among graded fertility bulls, despite the fact that some of the CTCF binding sites within DMR were varied (Jena et al. 2014). They also suggested that in order to reach a definitive result, bull fertility concerns should be addressed at a genome-wide level rather than at the level of individual genes.

There is a considerable body of data that epigenetic alterations in the sperm genome (both histone modifications and DNA methylation) influence its participation in early embryogenesis (Aston and Carrell 2009; Hammoud et al. 2009b). These findings support the idea that methylation deficiencies in both imprinted and non-imprinted genes, as well as other epigenetic anomalies (such as histone localization or modifications), have a role in spermatozoa fertilization ability. As a result, a full understanding of epigenetics in gametes and embryos based on the information gleaned from these findings could be extremely useful in reversing pathological states and restoring natural equilibrium. Furthermore, as indicated in earlier research, spermatozoal DNA methylation appears to be an informative parameter not only for spermatogenesis, but also for following fertilization events such as pregnancy success and outcome (Benchaib et al. 2005; Zheng et al. 2011; Ankolkar et al. 2012; Rotondo et al. 2012; Kumar et al. 2013; Lambrot et al. 2013).

Furthermore, only a few studies have examined the relationship between DNA methylation and histone alterations. Certain histone changes in chromatin impede DNA methylation, according to Ooi et al. (2007). (Ooi et al. 2007). Even differentially imprinted alleles have variable histone modifications in somatic cells that promote activation or repression (Delaval and Feil 2004; Delaval et al. 2007). In concordance, H3K4me3 was found to be related with the paternally expressed DMRs, whereas maternally imprinted (paternally repressed) loci lacked H3K4me3 and had moderate levels of H3K9me3, a repressive chromatin signature (Hammoud et al. 2009a, b). Moreover, Carvalheira et al. (2019) found that H3K27me3 immunolabelling for bovine blastocysts was embryo sex-dependent and bull-dependent (i.e., epigenetic), thus indicating paternal inheritance (Carvalheira et al. 2019). These nucleosome alterations contribute to the establishment and maintenance of parent-of-origin identity through epigenetic cellular memory. Male infertility cases with abnormal histone retention may disrupt this memory; however, it is unclear whether these modifications are to blame for poor embryo outcome in infertility patients with altered histone to protamine ratios.

In bovine oocytes there was a noticeable difference in methylation levels of in vitro (29.0%) and in vivo matured oocytes (31.6%) (Duan et al. 2019b). This difference indicates aberrant methylation pattern during in vitro maturation of oocytes, which could explain the link between in vitro maturation, fertilization, and culture leading to abnormal embryo development and gene expression (Smith et al. 2005, 2009) and large offspring syndrome (Young et al. 1998). Oocyte-specific linker histone, H1foo overexpression could stimulate extrusion of the first polar

body, suggesting its indispensible role during bovine oocyte maturation (meiotic progression) (Yun et al. 2015).

16.3.1 Epigenetic Potential of Nuclear Proteome in Relation to Fertility

Histone alterations, in addition to methylation, have been linked to infertility cases in both humans and animals. Azpiazu et al. (2014) discovered high quantities of proteins contributing to altered sperm epigenetic fingerprints in infertile individuals using high throughput differential proteomics in human male infertility cases. Besides, the infertile patients had lower levels of proteins involved in metabolism, particularly lipid/lipoprotein metabolism, implying that epigenome has a direct impact on metabolome (Azpiazu et al. 2014). The authors discovered that these proteome epigenetic changes could be the cause of infertility. Intriguingly, Vieweg et al. (2015) suggested that inappropriate histone acetylation, rather than DNA methylation, was the source of insufficient chromatin compaction, which they believed was the cause of spermatozoa's inability to transfer correct epigenetic marks to oocytes, resulting in infertility/subfertility (Vieweg et al. 2015).

Furthermore, new research has discredited the notion that spermatozoa exclusively serve as a carrier of DNA methylation to the next generation. It has been discovered that when sperm substitutes histones for protamines, a small subset of histones is maintained, which likely reduces the dominating maternal influence to some extent (Hammoud et al. 2011). According to these investigations, it is a typical programmed procedure rather than an anomaly. These histones, as well as their changes, serve as carriers of epigenetic information from generation to generation. The H3K4me2, H3K4me3, H3K27me3, and a testes-specific histone H2B (TH2B) (Kimmins and Sassone-Corsi 2005) are among the retained histones, which are subjected to various modifications such as methylation, acetylation, phosphorylation, ubiquitination, and ribosylation to facilitate chromatin access in a time- and space-specific manner. (Bannister and Kouzarides 2011; Petty and Pillus 2013). In a study by Kutchy et al. (2017), TH2B was found to be negatively correlated with the fertility of bulls, indicating its decrease is favorable for fertility in high fertile bulls (Kutchy et al. 2017). Verma et al. (2015) investigated two such modifications, H3K4me2 and H3K27me3, and discovered that genes involved in germ cell development, spermatogenesis, and embryonic development were differentially enriched between high and low fertility bulls, emphasizing their importance in bull reproductive performance (Verma et al. 2015). In humans, H3K4me2 is suggested as a biomarker for assessment of fertility, which was based on the negative correlations found between H3K4me2 and sperm concentration, motility, and mitochondrial activity (in normozoospermic, asthenozoospermic, and oligoasthenozoospermic samples) indicating aberrant histone-protamine exchange, resulting in improper chromatin condensation (Štiavnická et al. 2020).

16.3.2 Clinical Significance of Abnormal Protamine Expression

There is an evident link between abnormal protamine expression and infertility (Oliva 2006). In diverse species, insufficient protamine message, particularly with respect to P2, has been reported to induce oligozoospermia and DNA damage (Carrell and Liu 2001). According to the aforementioned remark, a high P1/P2 ratio is most likely the main cause of infertility in these circumstances (Aoki et al. 2006c). Infertility is not always caused by P2, as some recent data have identified dysregulation of the P1 gene in cases of infertility. Nevertheless, the P2 aberrations are more common in infertility situations, which are likely owing to the fact that the P2 gene evolved later than the P1 gene, making it more susceptible to modifications or variants (Lewis et al. 2003). The functional parameters of the mammalian spermatozoa that are affected by anomalous protamine expression include motility, acrosome integrity, DNA integrity, penetration ability, and others, all of which affect spermatozoa's fertilizing capacity, yet pregnancy rates were not different in ICSI-treated cases compared to IVF (Aoki et al. 2005, 2006a). It's thus possible to deduce that (a) aberrant protamine expression is more common in infertile men and (b) it's linked to severe spermatogenic abnormalities. Experimental animal investigations involving reduced protamine expression have supported up this interpretation. It has been discovered that abnormal protamine expression increases the rate of apoptosis, which could explain how the former causes poor spermatogenesis (Cho et al. 2001; Cho et al. 2003). Protamine insufficiency has been linked to DNA damage in mice, resulting in poor spermatogenesis. Protamine concentration of sperm and DNA damage have been found to be tightly linked in bovines as well. The reduced protamine levels are believed to cause DNA instability and damage, which can decrease bull fertility (Fortes et al. 2014). In a later study, It was observed that inadequate amount and localization of PRM1 were associated with defects in sperm chromatin condensation, coinciding with reduced fertility in bulls (Dogan et al. 2015). Rahman et al. (2011) showed higher level of protamine-deficient spermatozoa in Holstein-Friesian and Belgian Blue bulls, which were exposed to a testicular heat by scrotal insulation, results also coincided with abnormal morphology of sperm with presence of head abnormalities and nuclear vacuoles (Rahman et al. 2011). In another study with 40 nellore bulls, a negative association between bull age and protamine deficiency was evident. This indicated younger bulls (Mean: 1.9 years) are in an emerging state of spermatogenic maturity, evident from higher level of DNA fragmentation and teratospermia as compared to older bulls (Carreira et al. 2017). Kipper et al. (2017) conducted a study on same group of bulls, observed sperm head was morphologically larger in the younger bulls (Kipper et al. 2017). A large study on genetically well-defined group of tropically adapted bulls (Brahman and Tropical Composite) showed correlation between sperm DNA integrity and protamine deficiency (Boe-Hansen et al. 2018). In this study, presence of head shape alterations, cytoplasmic proximal droplets, and spermatogenic immature cells was correlated with both sperm DNA integrity and protamine deficiency. On the other hand, the alterations in protamine levels could potentially be the result of anomalies

in transcription, translation, or other phosphorylation pathways, which impact spermatogenic genes, including the protamines. Because of their roles in transcriptional and translational control, the Y-box proteins Translin and Contrin, as well as the related kinesin KIF 17B, may be of particular interest in this scenario. Other critical regulatory systems, such as phosphorylation pathways, could be implicated in spermatogenesis deficiencies broadly (Aoki et al. 2006c).

16.3.3 ART Failures and Associated Epigenetic Alterations

Many diseases have been linked to changes in imprinted gene expression (Jaenisch and Bird 2003; Seitz et al. 2004; Morgan et al. 2005; Royo et al. 2006). The Beckwith–Weidemann syndrome (BWS) and Angelman's Syndrome (AS) are two of these disorders that have been linked to neuromuscular defects in IVF offspring (Buiting et al. 2003; DeBaun et al. 2003; Gosden et al. 2003; Maher 2005). As reported by Houshdaran et al. (2007), the infertile patients with poor semen characteristics show a larger shift in DNA methylation in sperm at numerous imprinted loci, CpG islands upstream of gene promoters, and a few repetitive sequences (Houshdaran et al. 2007). Differences in sperm DNA methylation levels within specific genes or alleles suggested the possibility of a link between sperm DNA methylation and embryo health, as well as the presence of a disease risk threshold. Kagami et al. (2007) found that an ART born offspring with Silver–Russell syndrome (SRS) may have inherited aberrant methylation at the DMR of MEST in sperm (Kagami et al. 2007). Kobayashi et al. (2009) found aberrant DNA methylation at numerous imprinted sites in 17 of 78 assisted reproductive technology (ART) embryos studied in a more detailed research. The hypomethylation at H19 and GTL2 was found in both the sperm and the embryo in seven cases, showing that aberrant hypomethylation may be inherited from the father (Kobayashi et al. 2009).

16.3.4 Epigenetic Changes in Ageing Oocytes and Their Poor Fertilizing Ability

The term "ageing oocyte" refers to the ageing process that occurs in the ovaries of females during later phases of their reproductive cycle, resulting in a decline in oocyte number and quality, as well as a lower conception rate (Tatone et al. 2008). Studies that have attempted to understand the underlying phenomenon of such modifications have linked it to the creation of epigenetic alterations in the germline that can be transmitted to future generations. As a result, epigenetic changes that occur during oocyte maturation have a significant impact on oocyte quality.

16.3.5 Ageing Oocytes and DNA Methylation

The process and methods of methylation establishment in oocytes are similar to those in the spermatozoa. The erasure of DNMT1, a de novo methyltransferase, in embryos resulted in the loss of imprinting markers, leading to the loss of the produced embryos (Howell et al. 2001). In the oocytes of young and old mice, distinct patterns of methylation have been discovered. Furthermore, methylation has been seen to change in aged mice's oocytes and pre-implantation embryos. It is well-known that the older mice have a lower pregnancy rate than younger mice and have more severe foetal abnormalities, which could be linked to aberrant DNA methylation in oocytes (Yue et al. 2012). However, no clear evidence has been presented to link abnormal DNA methylation in oocytes with ageing. There are also studies that show that neither the monoallelic expression of genes like H19 and SNRPN nor the DNA methylation patterns of differentially methylated regions of imprinted genes in embryos produced from oocytes of ageing mice (Lopes et al. 2009) are altered in embryos produced from oocytes of ageing mice. Even the genome-wide methylation investigations in embryos and placentas of elderly female mice revealed no methylation abnormalities (Lopes et al. 2009).

16.3.6 Ageing Oocytes and Histone Modifications

Apart from DNA methylation, another prominent and well-studied epigenetic alteration is histone modifications, as previously stated. Histone deacetylases deacetylate the histones during the meiosis I and II. Any hindrance or alteration in this mechanism causes the fertilized oocytes to become aneuploid, resulting in embryonic mortality during the early stages of development (Akiyama et al. 2006). Interestingly, the histone acetylation, like DNA methylation, has been discovered to differ between elderly and young female mice (Suo et al. 2010). For example, the expression of Sirt 2, which is linked to histone acetylation (H4K16), was shown to be lower in older mice's oocytes than in younger mice's (Zhang et al. 2014). Furthermore, older female GV and MII oocytes were shown to be deficient in H3K9me3 when compared to younger female GV and MII oocytes (Manosalva and Gonzalez 2009). Besides, the H3K4 methylation in aged GV oocytes in mice has also been reported to alter (Shao et al. 2015). Recent research has discovered that histones in early stage oocytes (GV) are heavily acetylated; nonetheless, the histones in later stages of MII are variably deacetylated. Furthermore, histone acetylation was found to be related to maternal age, with older females having decreased H4K12 deacetylation in MII oocytes. Besides, the ubiquitination process was also found to be affected in older females. In such females, ubiquitin itself was downregulated, whereas the expression of ubiquitin-specific peptidases was increased (Grondahl et al. 2010).

16.4   Conclusions and Way Forward

Epigenetics, as previously mentioned, is a phenomenon influenced by dietary and environmental factors. These factors can have a significant impact on histone acetylation and DNA methylation. The intracellular dietary cofactors such as NAD+, acetylcoA, and ATP, among others, play a key part in this process. The folate-methionine pathway also provides methyl donors for DNA methylation, such as S-adenosylmethionine (Daniel and Tollefsbol 2015). As a result, the availability of dietary methyl donors and cofactors is crucial during development. Nutrition has an impact on DNA methylation during development, as evidenced by multiple studies that demonstrated that folate treatment can successfully avoid an abnormal epigenetic state caused by decreased DNMT1 activity (Lillycrop et al. 2005; Lillycrop et al. 2007). A permanent repression of IGF2 has been seen in methyl donor-deficient diets (Waterland et al. 2006). The epigenetic patterns created by these chemicals must be elucidated and described in order to provide a reliable diagnosis and future prognosis (Egger et al. 2004). They can also be used as new pharmacological targets or for the development of novel dietary combinations. Based on the evidence presented above, it appears that dietary and other environmental factors should be investigated further in relation to various epigenetic states in the search for novel drugs and molecules that may be able to restore the natural epigenetic balance in diseased individuals, particularly those with disturbed spermatogenesis.

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Conclusion

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Abstract

Bovine fertility is a complex trait that is influenced by several factors. Worldwide, the reproductive performance has decreased in cows with high genetic potential for milk production. The scope for improving fertility using genetics is challenging because of low heritability. In the given situation, a pragmatic approach could help to understand thoroughly the physiological and metabolic changes in high yielding bovines and to evolve suitable management and therapeutic strategies to maintain high fertility in high yielding bovines. Recent developments in reproductive bio-techniques have revolutionized and opened up new avenues for manipulating the reproductive process in bovines, both in vitro and in vivo, for improving their reproductive efficiency. The potential of these technologies is to be harnessed to achieve optimum reproductive efficiency in bovines.

Keywords

Declining fertility · Fertility concepts · Reproductive technologies · Bovines

Although differences in opinion exist among researchers, a majority of the studies indicate a decreasing trend of fertility in bovines. There is a need to develop tools/ methods to identify the inherent fertility of an animal because identification of animals with lower potential fertility prior to breeding would allow producers to

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make informed economic decisions regarding insemination. One of the reasons suggested for reduced reproductive efficiency of high yielding animals is alterations in metabolic adaptation and reproductive physiology due to intense selection for high milk production. However, on the other hand, some of the highest yielding herds in few countries are able to maintain excellent fertility, indicating that the negative association of high milk production with fertility can be minimized by adopting proper management conditions. The role of metabolic hormones on gonadal functions and fertility is immense, and therefore, a sound nutritional programme is to be implemented to prevent the adverse effects of negative energy balance on reproduction. Oestrus detection efficiency and accuracy of the herd should be maintained at a higher rate, which is possible when techniques with high sensitivity and specificity are employed for oestrus detection. In this direction, salivary fern pattern and oestrus specific salivary molecules offer ample scope to develop cow-side tools/tests for oestrus detection. In herds where oestrus detection efficiency is low, application of timed artificial insemination protocols can be beneficial. In the recent past, several protocols have been developed and/or modified to allow timed inseminations so as to circumvent the practical difficulties associated with oestrus detection. Implementing protocols that synchronize ovulation with the time of artificial insemination would give an added advantage in terms of high chances of conception. Nevertheless, it should always be remembered that inseminating cows with spermatozoa from high-fertile bulls is essential to achieve high conception rates. Further, it should also be ensured that the semen used for insemination of cows should be of high quality and the sperm fertilizing potential is preserved after cryopreservation. Use of high-throughput semen analysis techniques such as Computer Assisted Semen Analysis and Flow Cytometry for assessment of sperm kinematics and functionalities, respectively would help assessing the sperm fertilizing potential. Subclinical uterine infection adversely impacts the reproductive efficiency of bovines, therefore proper transition cow management protocol needs to be applied to downsize its incidence. During the last few decades, several new concepts have emerged in the field of bovine reproduction, which definitely advanced our understanding about bovine fertility and helped to evolve a number of technologies to maximize the reproductive efficiency. As a continuation of the current book, the technologies for improving bovine reproduction are detailed in a separate book on "Frontier technologies in bovine reproduction".