Sustainable Agriculture Reviews 59

Vinod Kumar Yata
Ashok Kumar Mohanty
Eric Lichtfouse *Editors*

Sustainable Agriculture Reviews 59

Animal Biotechnology for Livestock Production 3

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Sustainable agriculture is a rapidly growing field aiming at producing food and energy in a sustainable way for humans and their children. Sustainable agriculture is a discipline that addresses current issues such as climate change, increasing food and fuel prices, poor-nation starvation, rich-nation obesity, water pollution, soil erosion, fertility loss, pest control, and biodiversity depletion.

Novel, environmentally-friendly solutions are proposed based on integrated knowledge from sciences as diverse as agronomy, soil science, molecular biology, chemistry, toxicology, ecology, economy, and social sciences. Indeed, sustainable agriculture decipher mechanisms of processes that occur from the molecular level to the farming system to the global level at time scales ranging from seconds to centuries. For that, scientists use the system approach that involves studying components and interactions of a whole system to address scientific, economic and social issues. In that respect, sustainable agriculture is not a classical, narrow science. Instead of solving problems using the classical painkiller approach that treats only negative impacts, sustainable agriculture treats problem sources.

Because most actual society issues are now intertwined, global, and fast-developing, sustainable agriculture will bring solutions to build a safer world. This book series gathers review articles that analyze current agricultural issues and knowledge, then propose alternative solutions. It will therefore help all scientists, decision-makers, professors, farmers and politicians who wish to build a safe agriculture, energy and food system for future generations.

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Preface

Animal biotechnology has been used to produce elite animal breeds that have specific qualities such as improved growth rate, disease resistance, and improved milk and meat production. This third volume of the book on 'animal biotechnology for livestock production' focusses on proteomics and metabolomics approaches to develop novel methods of livestock production. This book reviews recent developments on animal reproduction.

Chapter 1 summarizes reproductive biotechnologies in female sheep and goats. Biotechnological methods such as in vitro embryo product, cloning and transgenesis are discussed in detail.

Chapter 2 discusses various aspects of oogenesis and folliculogenesis such as formation, growth, and development of follicles, and influence of the antral follicle count on the reproductive performance. This chapter also discusses epigenetic modifications during folliculogenesis.

Chapter 3 reviews the viral proteomics studies on most common animal viruses broadly categorized as animal viruses, human viruses and zoonotic viruses, to understand the role of proteins altered during viral infection. This chapter provides a list of proteins altered during virus infections and their probable role in viral pathogenesis.

Chapter 4 describes the mechanism of maintenance of ovarian reserve and oocyte quality in livestock species. Furthermore, this chapter also discusses the impact of ovarian disorders on livestock follicle reserve, and biotechnological interventions to improve oocyte reserve and health.

Chapter 5 focusses on applications of metabolomics in livestock, processed food, human nutrition and plant resource food. This chapter also discusses challenges in food metabolomics research, such as sample preparation, data analysis and identification of unknown compounds.

Chapter 6 reviews the selected photopositive compounds on ovarian functions in mammals. The molecular mechanisms of bioactive compounds such as kaempferol, quercetin, myricetin and resveratrol are discussed.

Chapter 7 presents recent developments in buffalo embryo technologies such as multiple ovulation and embryo transfer, in vitro embryo production, somatic cell nuclear transfer, and intracytoplasmic sperm injection.

Experimental dairy herd at ICAR-National Dairy Research Institute, India

The editors of this book would like to thank all the authors for contributing high-quality chapters. We also extend our thanks to the reviewers for their critical comments on the manuscripts. We express our sincere thanks to production team at Springer Nature for quick and efficient publication of this book.

Karnal, Haryana, India — Vinod Kumar Yata

Mukteswar, Uttarakhand, India — Ashok Kumar Mohanty

Marseille, France — Eric Lichtfouse

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About the Editors

Vinod Kumar Yata is an interdisciplinary researcher working at the National Dairy Research Institute, Karnal, India. Previously, he worked as an assistant professor in the Department of Biotechnology, Dr. B R Ambedkar National Institute of Technology Jalandhar, Punjab, India. He received his PhD in biotechnology from the Indian Institute of Technology Guwahati. Vinod specializes in interdisciplinary research which includes nanotechnology, microfluidics, animal biotechnology, cancer biology and bioinformatics. He has developed a microfluidic device for the separation of live and motile spermatozoa from cattle semen samples. His research interests have been focussed on the development of nanocarriers, understanding prodrug enzyme therapy and targeted drug delivery. He elucidated the structural features and binding interactions of several biomolecules by in silico methods. Vinod has published four books as editor and one book as author with Springer Nature. He has published several research papers in peer-reviewed international journals and presented papers in several international conferences.

Ashok Kumar Mohanty is an eminent scientist in the field of animal biotechnology and is currently serving as joint director of ICAR-Indian Veterinary Research Institute, Mukteswar, Uttarakhand, India. His group is involved in various basic and applied research related to animal production systems. His research group has made pioneering contributions in the field of animal biotechnology, with emphasis on gene cloning, expression and functional characterization of animal proteins, proteomics in animal production, cell and molecular biology, and structural biology of proteins. His group has developed a buffalo mammary epithelial cell line for the first time, which can be used as a model system to understand lactation biology in animals as well as humans. His team has also developed a pregnancy diagnostic kit for the early detection of pregnancy cattle and buffalo. His group is also extensively involved in developing low-cost technology for semen sexing in cattle. Ashok has organized a number of national and international workshops and international conferences. He is a recipient of several awards, which includes DBT Overseas Associateship by the Ministry of Science & Technology, Govt. of India., and Jawaharlal Nehru Award (gold medal) by the Indian Council of Agricultural Research (ICAR), New Delhi, for outstanding postgraduate research in the field of Animal Biotechnology. He is a fellow of the National Academy of Dairy Sciences (NADS), India, and executive member of proteomics society of India and associate fellow of the National Academy of Veterinary Sciences, India. He has supervised more than 50 graduate, PhD students and postdocs. Ashok has published more than 200 peer-reviewed research and review papers. He has also authored eight book chapters in the areas of animal and food biotechnology published by national and international publishers.

Eric Lichtfouse is Professor of Environmental Sciences and Scientific Writing at Aix Marseille University, Xi'an Jiaotong University and the University of Shanghai for Science and Technology. He has invented carbon-13 dating, a molecular-level method allowing to study the dynamics of organic compounds in temporal pools of complex environmental media. He has discovered temporal pools of individual substances in complex media such as soils. He is chief editor and founder of the journal *Environmental Chemistry Letters*, and the book series *Sustainable Agriculture Reviews*. He is the author of the book *Scientific Writing for Impact Factor Journals*. Eric has received awards in the fields of analytical chemistry and scientific editing. In sports, he is XTerra Vice-Champion in his age category.

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Chapter 1
Reproductive Biotechnologies Applied to the Female Sheep and Goat

Joanna M. G. Souza-Fabjan, Bruna R. C. Alves, Ribrio I. T. P. Batista, Alexsandra F. Pereira, Luciana M. Melo, Vicente José F. Freitas, and Maria Emilia F. Oliveira

Abstract Applying reproductive biotechnologies to female sheep and goats allows to improving animal protein production, advancing scientific knowledge with the use of small ruminants as animal models; and developing innovative biomedicine technologies. Multiple ovulation and embryo transfer (MOET), *in vitro* embryo production (IVP) and embryo sexing and cryopreservation, are some of the biotechnologies available for use. The control of the estrous cycle, the prediction of multiple ovulation results, and the difficulty in performing non-surgical embryo recovery procedures are the major chokepoints in MOET programs. Whereas oocyte quality and efficiency upon the use of defined media is a concern in IVP. Embryo survival after cryopreservation should be strengthened, especially in IVP embryos. More advanced reproductive biotechnologies, such as cloning by somatic cell nuclear transfer (SCNT), are increasingly being conducted in small ruminants. SCNT is a possible step for the production of transgenic animals, however, more advanced

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resources for performing transgenesis are being developed, and were already applied to small ruminants.

Here, we review reproductive biotechnologies in female sheep and goats. Deeper understanding on the control of estrous cycle and progress regarding the non-invasive transcervical embryo recovery have been achieved. Regarding IVP, the current outcomes reached in small ruminants are similar to the results obtained in bovine, allowing a safe application of this embryo production technique for commercial ovine and caprine production systems. Importantly, significant advancement has been achieved regarding survival after cryopreservation and establishment of pregnancy, especially in MOET-derived embryos. Regarding SCNT, attempts have been focused both on establishing the appropriate conditions for obtaining karyoplasts, cytoplasts, and on performing adjustments before and after embryonic reconstruction. For performing transgenesis, the easy and non-expensive molecular-based approaches, such as the CRISPR/Cas9 system, were made accessible to many research groups and industry, and had undoubtfully revolutionized the production of bioreactor animals.

Keywords Cloning · Cryopreservation · *in vitro* embryo production · *in vivo* embryo production · IVF · MOET · SCNT · Superovulation · Transgenesis · Vitrification

Abbreviations

ABE	Adenine base editors
ApAFP914	*Anatolica polita*-derived antifreeze protein
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR-associated system
COCs	Cumulus-oocyte complexes
DNA	Deoxyribonucleic acid
eCG	Equine chorionic gonadotropin
FSH	Follicle stimulating hormone
IVP	*In vitro* embryo production
MII	Metaphase II
mRNA	Massager ribonucleic acid
microRNA	Micro ribonucleic acid
lncRNA	Long non-coding ribonucleic acid
MOET	Multiple ovulation and embryo transfer
PCR	Polymerase chain reaction
pFSH	Porcine follicle stimulating hormone

RNA-seq	RNA sequencing
scRNA seq	Single cell RNA-seq technique
SCNT	Somatic cell nuclear transfer
TALENs	Transcription activator-like effector nucleases
ZGA	Zygotic genome activation
ZFNs	Zinc finger nucleases

1.1 Introduction

Small ruminant farming was one of the first agricultural activities developed by humans, as evidenced by paintings found in prehistoric caves. Nowadays, goats and sheep are still important sources of meat, milk, skin, wool, and fibers worldwide; and a growing concern regarding improving reproductive efficiency of those species exists. Efficient reproduction results in the production a larger number of healthy offspring in the shortest possible interval. Reproductive biotechnologies potentialize dispersion and multiplication of genomes from superior animals: those that produce more protein using less resources, and generating less environmental impact. Thus, combining high reproductive performance, with the use of reproductive biotechnologies, robustly supports accelerated genetic improvement and, consequently, higher productivity. Sheep and goats have been also playing a prominent role as animal models in biomedical research, due to their reasonable size, ease of handling, well-known estrous cycle characteristics, and relatively low-cost husbandry (Bertolini et al. 2016). In addition, because sheep and goats are food-producing species, the easy access to slaughterhouse-harvested ovaries widens the possibility of their use as models for general reproductive biology research, especially regarding oocyte physiology and *in vitro* embryo production. Studies on those topics are fundamental for supporting improvement of assisted reproductive techniques, which are largely employed to overcome some fertility issue in humans, and to help in the conservation of endangered species. Indeed, small ruminants' ovaries are highly similar to the human ovary, regarding its architecture and cortex consistency, supporting therefore its acceptability as models for reproductive biotechnology research (Fransolet et al. 2014).

The study of reproductive biotechnology focusing on the female sheep and goat is dated from the 1930s, aiming to address the question about the possibility of hybridism among both species (Warwick and Berry 1949; Warwick et al. 1934). The visualization for the first time of a sheep oocyte and a goat 4-cell embryo (which was auto-transferred to its donor) was detailed described, amid clear excitement from that research group, which finished their report announcing successful birth of two lambs and one kid, produced *in vivo* and transferred to recipients of the same species (Warwick and Berry 1949). Early work on artificial insemination in sheep (Iwanoff 1907), and initial research on cryopreservation of ovine semen (Bernstein and Petropavlovsky 1937) and, later, with caprine semen (Smith and Polge 1950) were conducted, and development of reproductive technologies on males and

females were complementary. Although many advances have been achieved regarding artificial insemination and semen technology in small ruminants (reviewed in: Lv et al. 2019; Salamon and Maxwell 2000), the focus of this chapter is to discourse on some background and current advances in the reproductive approaches applied to sheep and goats, with emphasis on oocyte and embryo-related technologies. The fascinating work leaded by Dr. Raymond Berry (Warwick and Berry 1949) inaugurated the research on embryo technology in farm animals and drove the attention to the "egg donor" female in the agriculture field. From investigations about reproductive physiology in mammals until a widespread utilization of embryo and oocyte donor at the commercial perspective in agriculture, and the establishment of the International Embryo Technology Society, years have passed (Betteridge 2003). Both *in vivo* and *in vitro* embryo production, later combined with embryo cryopreservation, manipulation, and bipartition, have been widely conducted in sheep and goats and, overall, those techniques have contributed to the elucidation of key events of mammalian embryo and pregnancy physiology, and supported advances in the agricultural industry of small ruminants. Importantly, those earlier-developed biotechnologies were foundational to the more advanced ones, such as cloning and transgenesis.

Of note, the species of choice for the first successful nuclear transfer of somatic cells (or cloning) in mammalian was the sheep (Wilmut et al. 1997), and the first cloned sheep, Dolly, is still acknowledged as the most famous laboratory-produced animal that has ever existed. This breakthrough shifted the attention of the research on reproductive biotechnology in sheep and goats, from the agricultural, to the pharmaceutical standpoint (Betteridge 2003). The high lactating potential of goats supported their use as bioreactors, which due to transgenic modifications, are able to secrete proteins of interest through their mammary gland (Bertolini et al. 2016). Hence, those small ruminant females are now perceived as putative "medicine makers". The accumulation of knowledge over the years, upon the contribution of countless researchers around the world, supported marked improvements of reproductive biotechnologies in small ruminants. The last-generation biotechnologies are certainly among the greatest outstanding discoveries and innovations in life-science of the last decades. The production of Dolly the sheep and animal bioreactor goats secreting human therapeutic proteins that are already commercially available are examples of such breakthroughs (Carneiro et al. 2018; Freitas et al. 2012). Therefore, this chapter aims to present, in the sheep and goat species, the main particularities, challenges, and latest improvements of the basic reproductive biotechnologies involving embryo production, as well as the last-generation approaches such as cloning and transgenesis, which derived from the former.

1.2 *In Vivo* Embryo Production

In vivo embryo production, also named multiple ovulation and embryo transfer (MOET), is a second-generation assisted reproduction biotechnology, which has enabled the rapid growth of sheep and goat herds around the world. In small ruminants, MOET has been the most used method for embryo production worldwide (Viana 2020). MOET programs aim to accelerate multiplication of superior genotypes, by increasing the number of offspring produced per estrous cycle of an ewe or doe and consequently, throughout her reproductive life. The use of this biotechnology favors the exchange of genetic material, especially over long distances. In addition, MOET is considered a critical tool for saving and maintaining endangered breeds and species, via establishment and expansion of germplasm banks. The most important steps of MOET programs are depicted in Fig. 1.1 and will be discussed as follows.

1.2.1 Donor Selection

Selecting females to be embryo donors in a MOET program is one of the most important aspects of this biotechnology. Choosing animals that have desired genotype and/or phenotype will guarantee acceleration and greater precision of the genetic improvement process. The criteria for donor selection might include genealogy, body shape, performance outcomes, and progeny test. The current technologies for identification of genomic markers had opened new possibilities for the early identification of superior individuals. Moreover, not only its genetic potential, but the donor's overall health and reproductive soundness are fundamental for determining success in MOET programs. In addition to evaluating dams' reproductive histories, some studies have suggested predicting their efficiency in responding to superovulation treatments. One strategy for this prediction is a pre-selection test, which comprises evaluation of the ovulatory response after a single dose of equine chorionic gonadotropin (eCG). This response was shown to correlate highly with further superovulatory responses induced by follicle stimulating hormone (FSH) treatments in sheep and goats (Balaro et al. 2016; Bruno-Galarraga et al. 2015). Likewise, antral follicular count and circulating anti-Müllerian hormone assessment has been considered suitable supporting strategies for selection of ewes with the greatest potential to respond to superovulation (Pinto et al. 2018; Balaro et al. 2016).

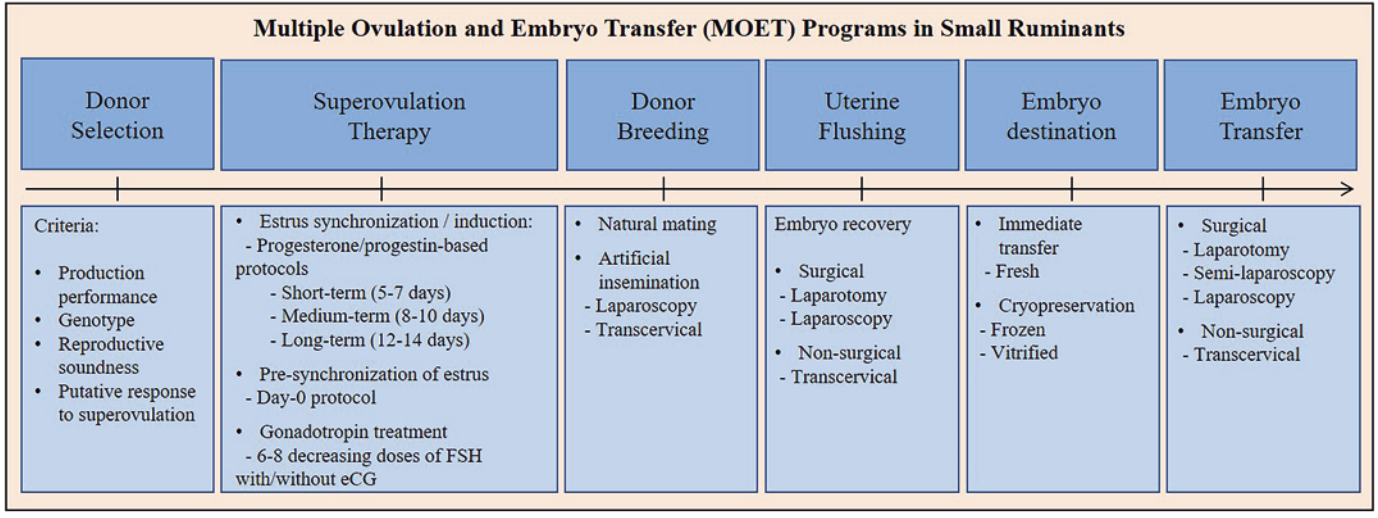

Fig. 1.1 Time line of the multiple ovulation and embryo transfer (MOET) programs and some peculiarities of each step in small ruminants, as it is approached throughout the chapter. *FSH* follicle stimulating hormone, *eCG* equine chorionic gonadotropin

1.2.2 Superovulation Therapy

Superovulation therapy involves two main activities: controlling the estrous cycle and inducing ovulation of multiple follicles (or superovulation). Estrous cycle control is performed by applying estrus synchronization/induction regimens. Hormonal superovulatory procedure is performed by administering elevated doses of gonadotropins. In sheep and goats, superovulation is one of the most arduous procedures, once the hormonal protocols are laborious and non-standardized (Oliveira et al. 2020; Bartlewski 2019; Camacho et al. 2019). Moreover, ovulatory responses and embryo yields are highly variable, and such unpredictability is the most limiting factor for application of superovulation in commercial herds (Oliveira et al. 2020; Bartlewski et al. 2008a; Gonzalez-Bulnes et al. 2004).

A series of factors, intrinsic and extrinsic to the donors, has been related to the high variability of the superovulatory response, such as: genotype, breed, age, reproductive status and history (number of births, postpartum interval, prolificacy, fertility, among others), reproductive season, nutritional plan, climate, flock management, gonadotropin preparations (commercial product, lot, dose and frequency administrations), insemination technique, and interval between consecutive treatments (Bartlewski et al. 2016; Gonzalez-Bulnes et al. 2004). Non-adequate superovulatory responses may be associated with several abnormalities of ovarian function, such as failure in follicular development and ovulation, luteinization of unruptured follicles, ovulation that does not result in corpus luteum, shortened corpus luteum lifespan, and ovulation of multiple follicles of varying developmental stages and sizes (Bartlewski et al. 2011). The consequences of these ovarian abnormalities are disrupted fertilization and inferior production of viable embryos. Therefore, to overcome those challenges, the treatment with gonadotropins, is often accompanied by other hormonal protocols for controlling timing of estrus, ovulation, follicular wave emergency, as well as circulating progesterone concentrations.

1.2.2.1 Hormonal Protocols for Controlling the Estrous Cycle

The most commonly applied method of controlling the estrous cycle for superovulation treatment in small ruminants includes intravaginal administration of a progestogen (progesterone or synthetic progestin) for 12–14 days (Fig. 1.2). Those long-term protocols have been designed according to the length of the luteal phase and result in satisfactory estrus induction, however variable fertility (Gonzalez-Bulnes et al. 2005; Menchaca and Rubianes 2004). Superovulatory regimen starts 2–3 days before the end of progestogen priming (Bartlewski et al. 2016; Oliveira et al. 2016a) (Fig. 1.2). Maintaining high circulating progesterone concentrations, from exogenous or endogenous source, is important for eliciting adequate follicular development and producing good-quality oocytes (Evans 2003). Even in cyclic females, protocols including progestogens are commonly used, either

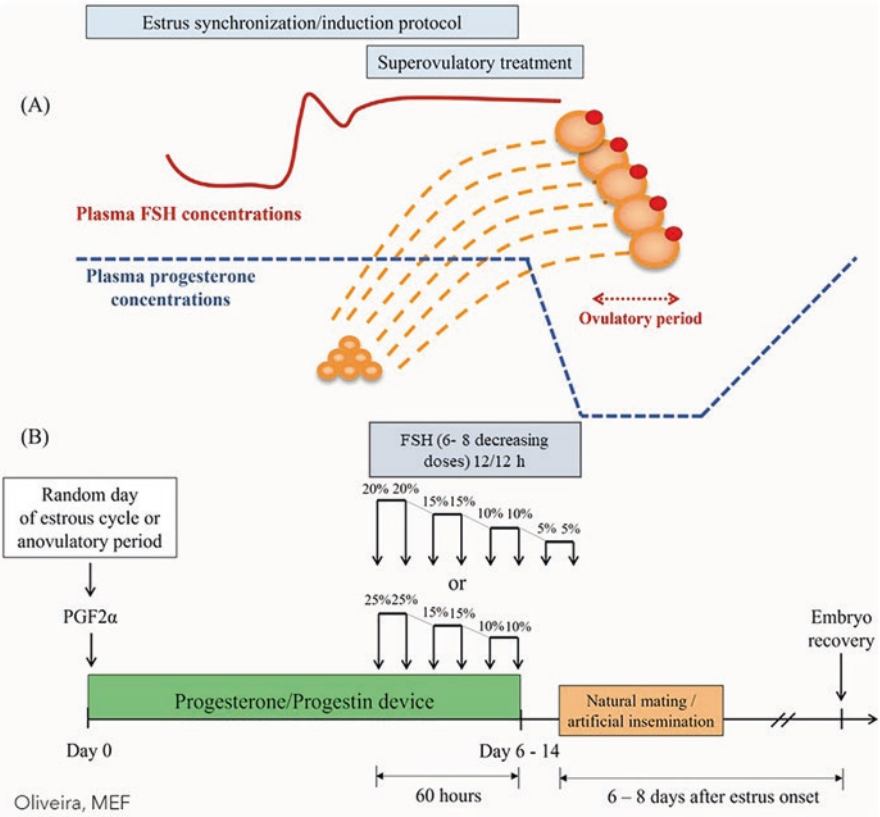

Fig. 1.2 Progesterone-based estrus synchronization/induction protocol followed to superovulatory treatment with follicle stimulation hormone (FSH), natural mating/artificial insemination, and embryo recovery in sheep and goats. The prostaglandin F2α (PGF2α) can be administrated at the beginning or the end of the progesterone protocol

concomitantly with gonadotropin treatment, or in the pre-synchronization of estrus, before superovulation. Progesterone or synthetic progestin resulted in similar outcomes regarding superovulatory response and embryo yield in ewes (Bartlewski et al. 2015) and goats (Camacho et al. 2019). In the Day 0 protocol (which will be detailed below), the administration of a progesterone device at the start of FSH treatment led to a greater oocyte developmental competence in sheep MOET programs (Cuadro et al. 2018; Menchaca et al. 2018). In those estrus synchronization protocols, administration of prostaglandin F2α is necessary, and can be done at the beginning (Day 0) or the end of the progestogen treatment. The treatment at the end of the protocol guarantees that any corpus luteum present will be sensitive to prostaglandin F2α (Camacho et al. 2019). Conversely, treatment on Day 0, guarantees a similar progesterone concentration profile between animals throughout the estrus induction treatment (Menchaca et al. 2007a).

Potential harmful effects of the prolonged progesterone treatment have been associated with a fast decrease in serum progesterone concentration before the end of the treatment (e.g. after approximately 10 days) (Oliveira et al. 2016a, b; Letelier et al. 2009). Sub-luteal circulating progesterone concentrations (<2 ng/mL) might modify the pattern of antral follicular emergence and growth, which negatively affects the subsequent events of ovulation, fertilization, and early embryonic development (Gonzalez-Bulnes et al. 2005). In more detail, sub-luteal progesterone concentrations during the induced luteal phase can result in the development of persistent follicles, which has been linked to precocious cumulus cells expansion, disrupted oocyte maturation, and abnormal embryonic development (Seekallu et al. 2010). Long-term progesterone pre-treatments can also result in a shorter interval to estrus (Harl 2014), and this can affect superovulatory response (Veiga-Lopez et al. 2008). Several studies point out alternative strategies to avoid sub-luteal concentrations of progesterone throughout the superovulation protocol, such as (1) administration of exogenous progesterone before removal of progestogen-soaked intravaginal devices (Hunsein and Ababneh 2008); (2) replacement of the progesterone device halfway through the duration of the treatment (Oliveira et al. 2012; Gonzalez-Bulnes et al. 2002); (3) medium-term (8–10 days in duration) or short-term (5–7 days in duration) protocols (Figueira et al. 2020a; Oliveira et al. 2014, 2020) (Fig. 1.2); and (4) Day 0 protocol, in which estrus pre-synchronization induces superovulatory treatment in the presence of at least one corpus luteum and near the first follicular wave emergence (Menchaca et al. 2009).

Progestogen supplementation 24 h before progesterone device removal can be used efficaciously for improving reproductive performance of ewes out of the breeding season (Hunsein and Ababneh 2008), however, this strategy has not been studied in superovulated ewes and goats. Replacement of progesterone device halfway (Day 7) through the 14-day estrus synchronization protocol resulted in reduced serum progesterone concentrations to less than 2 ng/mL on the last 3 days of the treatment, therefore, sustained a supraphysiological rise in circulating progesterone concentrations throughout the treatment period (Oliveira et al. 2016a, b). With the aim of reducing the length of progesterone priming, Merino ewes were exposed to 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14-day estrus synchronization protocols followed by a superovulatory treatment; which elicited similar embryo yield (Menchaca et al. 2009). This study showed that the length of the treatment could be more flexible and with practical advantages. In corroboration with this latter study, short-term (5–7 days) protocols have been proposed as alternatives to long-term protocols. Ovarian responses and embryo yields were similar between ewes submitted to short- (6 days) or long-term (12 days) progesterone-based synchronization protocol followed FSH treatment in eight decreasing doses (Oliveira et al. 2014). Similarly, a short-term (6.5-day protocol) progestin-based estrus synchronization treatment regimen followed by 3-day FSH treatment in the same breed of ewes resulted in similar superovulatory responses, except for the greater number of degenerated embryos compared with the long-term (14.5 days) progestin regimen plus a 4-day FSH treatment (Oliveira et al. 2020). The negative effect on embryo quality was related to the time of gonadotropic treatment, as discussed in the sequence of this

topic. In another study, a short-term (6 days) progestogen-based estrus induction protocol was compared to a medium-term (9 days) regimen for superovulation of Lacaune ewes, and the 9-day protocol resulted in better embryo yield (Figueira et al. 2020a). This result was expected since an increase in the number of follicles between 4.1 and 5.0 mm was observed in the last days of progestin treatment, followed by a higher number of ovulations and embryo yield in non-superovulated ewes submitted to 9-day estrus induction protocol, when compared to 6-day treatment (Figueira et al. 2020b).

The presence of a dominant follicle at the beginning of a superovulation protocol promotes adverse effects on other follicles and compromise the quality of oocytes and subsequent embryo yield. However, mid-cycle follicular wave emergency is not easily manipulated in sheep and goats, and the control or the synchronization of the emergence of a follicular wave is not performed in traditional superovulation protocols (Fig. 1.2). The vast majority (>75%) of sheep submitted to a traditional superovulation protocol were shown to have large follicles (≥5 mm) at the beginning of treatment with FSH (Menchaca et al. 2007b). The emergence of a follicular wave in sheep and goats occur at the time of ovulation (Evans et al. 2000) and ovulation can be timely-controlled. Based on this possibility, Day 0 protocols were proposed, setting the beginning of the superovulation treatment at the emergence of the first follicular wave of the estrous cycle (or at day 0 of the estrous cycle). Thus, the Day 0 protocol has a preliminary step: pre-synchronization of estrus and ovulation with one of the traditional protocols described above. The Day 0 protocol has been shown to promote an increased number of ovulations, fewer occurrences of anovulatory follicles, and premature luteal regression, as well as, a greater number of viable embryos compared to traditional treatments, both in sheep and goats (Menchaca et al. 2010) (Fig. 1.3).

Other strategies for controlling the follicular wave emergence have been proposed, but still without major applicability. Despite the long-term progesterone-based estrus synchronization protocols were not developed to synchronize follicular wave emergence in ewes, an expectable pattern of wave emergence during the 14-day treatment has been observed; however, follicular wave emergence timing was influenced by the number/frequency of follicular waves as well as ovarian status on the day of progesterone insertion (Oliveira et al. 2016a). In anestrous ewes, a single injection of estradiol-17β (350 μg/ewe) during the progestin protocol induced regression of large antral follicles and caused synchronous wave emergence ~5 days after injection (Bartlewski et al. 2008a). Thus, the synchronization of follicular waves with this hormonal combination before superovulation reduces the variability in superovulatory responses in seasonally anovulatory ewes (Bartlewski et al. 2008b). Despite that, synchronization of the emergence of the follicular wave with the combination of estradiol/estradiol ester and progesterone/progestin priming is not as effective, as it is in cattle. In cyclic ewes, a single injection of 350 μg estradiol benzoate 6 days before the first pFSH dose did not enhance the superovulatory response but increased the proportion of degenerated embryos by donor ewe (Oliveira et al. 2019).

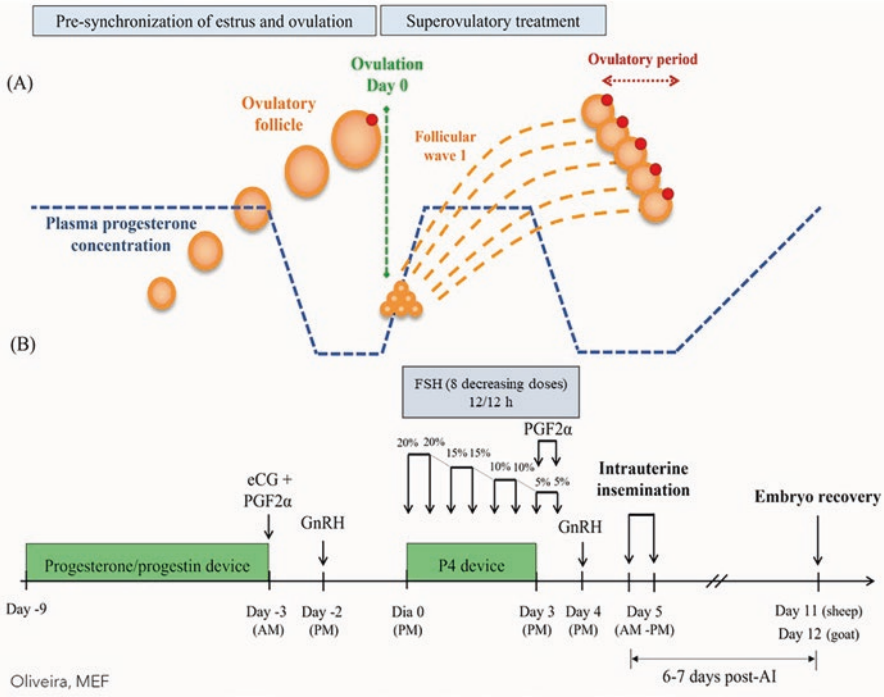

Fig. 1.3 Day 0 protocol for multiple ovulation and embryo production in sheep and goats. This strategy consists of pre-synchronization of estrus and ovulation, and consequently, of the emergence of the follicular wave 1. The superovulatory treatment with follicle stimulation hormone (FSH) starts near of emergence wave, and a new progesterone/progestin device remains inserted for 3 days. Prostaglandin F2α is administrated in two half doses together with the last two doses of FSH, and GnRH is given 24 h later to induce ovulations. Artificial inseminations are performed 16–24 h after GnRH and the uterine flushing is done 6–7 days after AI

1.2.2.2 Gonadotropin Treatment

Superovulatory treatment is based on hormonal preparations that aim to overcome the physiological follicular dominance, by stimulating development of a greater number of preovulatory follicle and, consequently, increasing number of ovulations (Blanco et al. 2003). Establishment of follicular dominance occurs, among other aspects, by depriving subordinate follicles of FSH (Ginther et al. 1996). Hence, to increase the number of ovulations and the number of embryos obtained by a treated female, a superovulatory treatment is carried out by the administration of high doses of gonadotropins, either FSH or eCG; or even FSH combined with eCG (Forcada et al. 2011; Pintado et al. 1998) (Figs. 1.2 and 1.3). Over the past few decades, the most used gonadotropin for the superovulation of sheep and goats is FSH derived from ovine or porcine pituitary extracts. For FSH treatment, the number and frequency of gonadotropin administrations, as well as the dose used may vary according to the protocol and influence the responses. FSH has a short half-life, requiring

administration at 12-hour intervals. In anestrous ewes, the FSH administrated at 08:00 and 16:00 h was more effective in terms of inducing multiple ovulations than the protocol with 12-hour intervals, but it was not associated with increased production of viable embryos by anestrous ewes (Bartlewski et al. 2017). This alternative FSH administration regimen has not been studied in cyclic ewes and goats.

Usually, the superovulatory treatment begins 60 hours before the progesterone/progestin device removal, and it is administered in fractional doses, during 3 or 4 days, totaling either six or eight applications, respectively (Figs. 1.2 and 1.3). Total FSH doses usually vary from 133 to 256 mg for sheep (Arrais et al. 2021; Figueira et al. 2020b; Oliveira et al. 2012, 2014) and from 120 to 200 mg for goats (Maia et al. 2020; Moura et al. 2010; Freitas et al. 2007). Decreasing doses of FSH are commonly used to mimic the endocrine changes in pituitary secretion during the follicular phase of the estrous cycle and tend to increase ovulation rate and embryo production (Gonzalez-Bulnes et al. 2004). Some findings pointed out that a 4-day ovarian superovulatory treatment regimen may result in increased follicular blood flow, which is detrimental to oocyte quality (Oliveira et al. 2014). A positive correlation between the follicular blood flow on the fourth day of the treatment regimen and the percentage of unfertilized eggs in ewes was also observed (Oliveira et al. 2014). In the same way, Oliveira et al. (2020) collected more degenerated embryos only from the ewes submitted to the 4-day pFSH treatment (eight doses) compared to the 3-day pFSH treatment (six doses). Moreover, besides being costly, high exogenous FSH doses might alter ovarian endocrine dynamics and luteinizing hormone pulse frequency, and induce prolonged stimulus of anovulatory follicles and early luteal regression (Loiola Filho et al. 2015; Okada et al. 2000).

Reduction in FSH doses for inducing sheep superovulation has been proposed. A total 100 mg dose of pFSH was suggested in the superovulation of Santa Inês ewes. The lower dose of pFSH (100 mg) resulted in similar numbers of ovulations and viable embryos than the 200 mg dose; however, a relatively greater percentage of apoptotic embryonic cells was observed (Maciel et al. 2019). A superovulatory treatment in ewes, with a total dose of 100 mg of pFSH, results in better corpora lutea development in the early luteal phase, and in more appropriate ovarian blood perfusion (Rodriguez et al. 2019). In lactating Lacaune ewes, 100 mg pFSH was not sufficient for eliciting expected superovulation responses, while 200 mg resulted in best superovulatory response and embryo recovery (Figueira et al. 2020c). Lactating females might require higher doses of FSH to promote superovulation due to their more intense metabolism. Although currently available protocols result in good superovulatory results, the biggest challenge remains the high variability between individuals or even between repetitions of MOET sections within the same donor. Studies have indicated that better results could be achieved with the customization of protocols according to season and breed. Additionally, the choice of the protocol should take into consideration the ovarian status of ewes at the time of progesterone/progestin device insertion (i.e., size of antral follicles and presence of luteal structures).

1.2.3 Donor Breeding

Breeding the female embryo donor might occur by natural mating or artificial insemination. The laparoscopic artificial insemination technique is highly used in superovulated sheep, whereas, in goats, artificial insemination by transcervical route is easier and efficient (Menchaca et al. 2010; Cognié et al. 2003). The cervical anatomy of the sheep is the major limitation for the transcervical approach (Fonseca et al. 2016; Kershaw et al. 2005). In superovulated ewes and goats, the recovery of unfertilized structures is common, and in some cases, implicates in low number of total and viable embryos. Incompetence of the oocytes to be fertilized, interference in the sperm transport due to the gonadotropin treatment, and asynchrony between the moment of breeding and ovulation (for example, lack of synchrony between the sperm and oocyte viability) are some of the possible causes of fertilization failure (Menchaca et al. 2010; Hyttel et al. 1991).

1.2.4 Embryo Recovery

Uterine flushing for embryo recovery can be performed in small ruminants by surgical, laparoscopic, or transcervical methods (Fonseca et al. 2016). In sheep, the most used technique is the surgical method, by laparotomy, while in goats the transcervical technique is easier and widely used (Camacho et al. 2019; Bartlewski et al. 2016). The surgical embryo recovery technique, although efficient, has a higher cost and might promote post-surgical disorders such as adhesions, limiting the number of times that the female can be repeatedly subjected to this procedure (Flores-Foxworth et al. 1992). Despite the cervical anatomy in sheep being the major choke-point for the use of non-surgical approaches in reproductive biotechnologies (Fonseca et al. 2016), drug-induced cervical dilation protocols, that facilitated transcervical uterine access and embryo recovery in ewes have been developed (Fonseca et al. 2019a, b, c). The non-surgical embryo recovery technique in sheep involves adequate training and the use of specific instruments. This technique has shown very encouraging results and great benefits to the animals. Importantly, drug-induced cervical dilation is a central step for non-surgical (transcervical) embryo recovery (Candappa and Bartlewski 2014). In recent studies, the association of d-cloprostenol, estradiol benzoate, and oxytocin supported high rates of cervical transposing, as well as successful uterine flushing, and higher percentage of fluid recovered in ewes (Fonseca et al. 2019a, b, c). Successful cervical transposing is affected by the complex cervical anatomy, and the difficulty of its visualization, clamping, and retraction (Fonseca et al. 2019a).

The steps for conducting transcervical uterine flushing in sheep and goats follow (Fonseca et al. 2019a): (i) application of the hormonal cervical dilation protocol; (ii) female restraining in a standing position, anesthesia and vulvar hygiene; (iii) cervical os visualization after insertion of a Collin specula into vagina; (iv) cervix clamping, laterally to the cervical os, with two Pozzi forceps; (v) traction of the cervical

canal; (vi) digital manipulation through the rectum and mapping the position of the cervical rings during cervical transposing by Hegar dilator; (vii) cervical transposing by a catheter guided by a stylet; (viii) total cervical transposition and removal of the stylet; and (ix) uterine flushing with phosphate buffered saline medium. Similar results were reported when nonsurgical (transcervical) or surgical (laparotomy) embryo recovery methods were compared in ewes (Santos et al. 2020). Likewise, equivalent results were achieved in repeated embryo collections by transcervical technique (Oliveira et al. 2020).

1.2.5 Achievements of In Vivo Embryo Production

Due to the efforts of researchers focusing on making MOET programs more feasible to small ruminant farmers, the number of embryos produced worldwide is increasing. The main critical factors that affect the expansion of commercial use of MOET in small ruminants are the unpredictability of the superovulatory response of each animal, the massive application of surgical embryo collection technique, and the elevated cost of protocols and procedures. The knowledge acquired in the recent years allowed the technicians to improve donor selection, and supported a deeper understanding on the control of estrous cycle, avoiding the presence of a large follicle at the start of FSH protocol. The importance of maintaining high progesterone concentrations throughout the entire treatment was highlighted, and a huge progress on the non-invasive transcervical embryo collection has been achieved. Hence, MOET may be widely acceptable regarding the constantly public concerns about animal welfare. Altogether, these factors may boost the expansion of commercial use of MOET in small ruminants.

1.3 *In Vitro* Embryo Production

In vitro embryo production (IVP) has increasingly been implemented in livestock genetic improvement programs. This technology has several advantages in comparison to MOET, such as its reliability and reproducibility. Oocyte donors for IVP might include prepubertal ewes and does, females that do not respond to superovulation, or culled ones, immediately after death. There has been a considerable increase in the number of IVP-derived embryos in sheep during the last years, however, compared to the bovine, the IVP industry in small ruminants is of lower relevancy (Viana 2020). Despite all the advantages of IVP, inconsistency in number and quality of harvested cumulus-oocyte complexes (COCs), as well as low embryo cryotolerance, still hamper the diffusion of this biotechnology worldwide (reviewed by Souza-Fabjan et al. 2021a; Paramio et al. 2020). *In-vitro* embryo production comprises four main steps (Fig. 1.4): (1) recovery and selection of high-quality

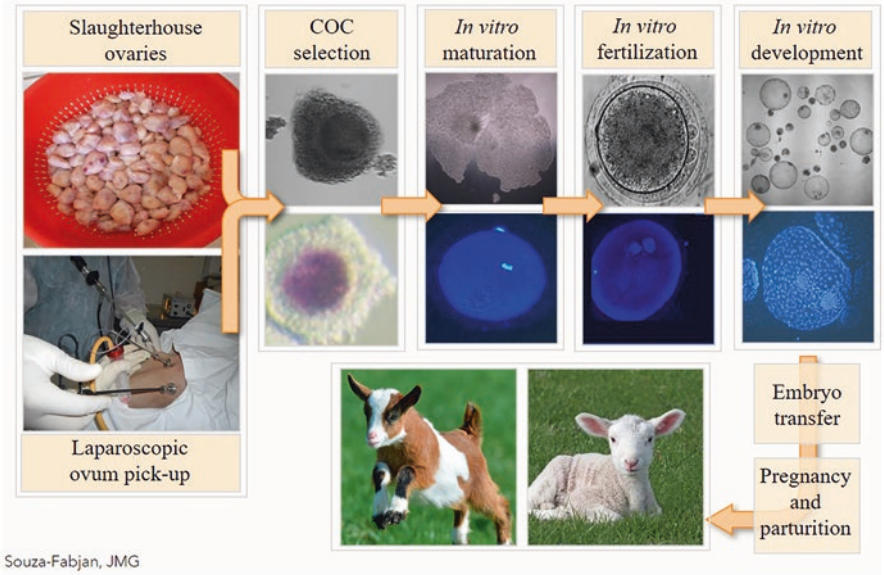

Fig. 1.4 Steps to perform *in vitro* embryo production in goats and sheep. The collection of cumulus-oocyte complexes (COCs) may be either from slaughterhouse reproductive tracts or by laparoscopic ovum pick-up. COC selection may be performed either morphologically (number of cumulus cells and ooplasm homogeneity) or aided by brilliant cresyl blue staining. Subsequently, Hoechst 33342 staining can be used to assess in meiotic maturation stage of the oocyte after *in vitro* maturation, the pronuclear formation after *in vitro* fertilization and the blastomeres cell count after *in vitro* development step. Blastocysts presenting high quality may be transferred to recipient females in order to the embryo continue its development, resulting in a healthy offspring

immature COCs; (2) *in vitro* maturation of the selected COCs; (3) *in vitro* fertilization of matured COCs, and (4) *in vitro* development (or culture) of embryos. Normally, small ruminant embryos are cultured *in vitro* until the compacted morula or blastocyst stage, on Day 6–8 after fertilization (Souza-Fabjan et al. 2019). Several steps of the IVP are also crucial for further developed biotechnologies, such as cloning and transgenesis (Baldassarre and Karatzas 2004), which will be discussed in the next sections.

1.3.1 Cumulus-Oocyte Complexes Collection and Selection

In live ewes and does, laparoscopy is the technique of choice for recovering high-quality cumulus-oocyte complexes (COC). In goats the transvaginal ultrasound guided ovum pick-up method (commonly used in cattle) has not shown to be as

successful as laparoscopy, according to one study (Graff et al. 1999). Recovery of COCs from females in the immediate *post-mortem* period (commonly at slaughterhouses), after removal of ovaries, and performing follicle aspiration or ovary slicing, is a possible alternative for COCs retrieval. This strategy permits obtaining COCs at lower costs, and often from a larger number of donors. Oocyte quality is the main factor affecting IVP results, seen as the production of high-quality blastocysts. Several aspects may contribute to the recovery of high-quality COCs, such as: female individual response to hormonal stimulation protocol, female sanity and nutrition, female age, season of the year, besides the operator experience, among others.

The preference for using ovarian stimulation by exogenous gonadotropins previously to COCs retrieval in small ruminants is practically unanimous, as it increases the number of pre-ovulatory follicles and, consequently, the number of COCs recovered per female. Several schemes of hormonal stimulation protocols have been tested and compared, but there is no consensus whether using either several (three to five) decreasing 12-hour apart doses of FSH or one-shot regimen (combination of one dose of FSH and one dose of eCG) has been achieved. In goats, five, in comparison with three doses of FSH, resulted in better quality of COCs, and with superior meiotic competence (Almeida et al. 2011). A recent alternative is found in the literature for COCs retrieval in sheep. It consists in the use of a Day 0 protocol for better synchronizing of the follicular wave, for the start of FSH administration (Bragança et al. 2018, 2021). In a first study (Bragança et al. 2018), four distinct gonadotropin treatments, all starting on Day 0, were compared: one-shot or three decreasing doses of FSH (totaling either 80 or 120 mg). The one-shot FSH treatments were combined with concurrent administration of eCG (300 IU). It was found that the lower dose of FSH was enough to promote the development of multiple follicles, resulting in the collection of COCs of good morphological quality. The three-dose regimen seemed to be more appropriate for producing better-quality oocytes, since markers of quality and steroidogenic pathway were upregulated in comparison with the non-stimulated control and the one-shot treatment, respectively (Bragança et al. 2018). In a following study, it was found that the supplementation of exogenous progesterone as intravaginal device at the time of FSH stimulation was better for supporting oocyte developmental competence, when compared to an analogue (progestin) or no device (Bragança et al. 2021).

An interesting approach for reducing the generation interval in goats and sheep is the use of prepubertal or juvenile females as oocyte donors. However, it is well known that COCs from prepubertal ewes and does have lower developmental competence (19–24%) when compared to adult-derived (34–51%) ones (Leoni et al. 2015; Leoni et al. 2009). The lower competence of prepubertal oocytes is strongly associated to the small size of follicles available in the ovary. There is also evidence that prepubertal females have an impaired antioxidant defense system, implicating in higher sensitivity of oocytes to detrimental effects produced by reactive oxygen species (Souza-Fabjan et al. 2021a). Regarding season, although it is possible to collect COCs from live females throughout the whole year, overall greater COCs developmental competence is achieved during the breeding season in small ruminants (Souza-Fabjan et al. 2021b; Mara et al. 2014).

1.3.2 Cumulus-Oocyte Complexes Selection and In Vitro Maturation

The only non-invasive method for detecting good-quality cumulus-oocyte complexes (COCs) after their retrieval is the evaluation of their morphology, which is also the most worldwide used method to select COCs in small ruminants (Fig. 1.4). This selection approach is usually performed following a grade classification either from 1 to 3 (Souza-Fabjan et al. 2016) or from 1 to 4 (Almeida et al. 2011). Those classifications take into consideration the number of cumulus cells layers and the cytoplasm homogeneity; and normally only Grades 1 and 2 (Souza-Fabjan et al. 2016) are chosen to proceed to *in vitro* maturation. Grade-3 oocytes, i.e., those partially or entirely denuded at collection, are commonly rejected. However, in goats, it was demonstrated that developmental competence of Grade-3 oocytes co-cultured with intact COCs during *in vitro* maturation, in equal proportions, was increased (Souza-Fabjan et al. 2016; Souza et al. 2013). Hence, co-culturing might be a useful approach to support the production of additional embryos from high-value does/ewes. It is noteworthy that grade selection is partially subjective, and a simple tool can be applied to make this selection more objective: the use of brilliant cresyl blue staining (Bragança et al. 2018, 2021) (Fig. 1.4). In sheep, COCs selected with the aid of this staining were more competent to develop up to blastocyst and produced embryos of higher quality (Catalá et al. 2011, 2012).

After being selected, COCs are washed and transferred to an *in vitro* maturation medium. Overall, for the *in vitro* maturation step, groups of approximately 50 COCs are incubated in four-well plates in 500 μL of medium, under 5% CO_2 in air at 38.5–39 °C, with maximum humidity, for 22–24 hours (Souza-Fabjan et al. 2019). During this step, COCs must undergo both nuclear and cytoplasmic maturation. The most commonly used maturation medium is Tissue Culture Medium-199, supplemented with different types of serum (undefined system), bovine serum albumin (semi-defined system) or totally-purified molecules (defined system) (reviewed by Souza-Fabjan et al. 2021a). Embryo development is highly affected by the events occurring during *in vitro* maturation. In laparoscopic-derived COCs, adding serum during *in vitro* maturation improved blastocyst rates; whilst a defined medium containing only epidermal growth factor as the protein source improved embryo production from slaughterhouse-derived COCs (Souza-Fabjan et al. 2013, 2014).

1.3.3 In Vitro Fertilization

In vitro fertilization comprises the coincubation of matured COCs and spermatozoa. Overall, for the *in vitro* fertilization step, groups of approximately 50 COCs are incubated in four-well plates, in 500 μL of medium, under 5% CO_2 in air at 38.5–39 °C, with maximum humidity for 16–20 hours (Souza-Fabjan et al. 2019). Either fresh or frozen–thawed semen can be used for fertilization. In the last decade,

lambs have been produced after the use of sex-sorted semen within the *in vitro* fertilization technique, with a reasonably high accuracy rate (87%) of the expected gender (Hollinshead et al. 2004). No data on sex-sorted semen is available in IVP in goat species yet. In order to select a high-motile spermatozoa population, performing assortment methods such as Percoll gradient centrifugation or Swim-up is necessary before proceeding with the coincubation. Generally, Percoll gradient centrifugation is used for frozen-thawed sperm selection (Rho et al. 2001) whilst Swim-up is preferable when using fresh semen (Palomo et al. 1999).

In small ruminants, spermatozoa capacitation is usually performed by the use of heat-inactivated serum from goat or sheep in estrus (estrous sheep/goat serum). The association of estrous sheep serum with heparin in the fertilization medium enhanced the blastocyst rate in goats (Souza et al. 2013). The sperm concentration varies expressively in the literature, but usually ranges between 1 and 2 million spermatozoa per milliliter. This parameter is strongly associated to the occurrence of polyspermy, since the increase in the number of spermatozoa and/or proportion of spermatozoa/COC may increase the polyspermy rates. Although this might not be a general rule, polyspermy was shown to be higher in goats than in sheep (Souza-Fabjan et al. 2021a). Regarding the presence of cumulus cells during the *in vitro* fertilization in sheep and goats, in addition to promoting the beneficial effect of increasing blastocyst rates (Dos Santos-Neto et al. 2020; Souza et al. 2013), these cells may also help as a physical barrier to the penetration of more than one spermatozoon.

1.3.4 In Vitro Development

After *in vitro* fertilization, presumptive zygotes are transferred to a culture medium that allows their development up to the blastocyst stage (day 6–8 post fertilization). Overall, for the *in vitro* development step, it is important to promote an embryo confinement, enhancing the diffusion of both autocrine and paracrine factors, which positively affect embryo development. To achieve this, small groups of approximately 20–25 presumptive zygotes are incubated in overlaid droplets of medium (1–2 μL per embryo), under 5% O_2, 5% CO_2, and 90% N_2, at 38.5–39 °C (Souza-Fabjan et al. 2019). Although different media have been used for embryo development in small ruminants, the most used is the Synthetic Oviduct Fluid, which was initially developed based on sheep oviductal fluid (Tervit et al. 1972). This basic medium is commonly supplemented with protein sources, such as bovine serum albumin, growth factors or fetal calf serum. However, it is well known that the use of high concentrations of fetal calf serum is strongly associated to the occurrence of the "large offspring syndrome" in ruminants (Rooke et al. 2007). The use of undefined systems (based on serum collected from diverse sources) has also the disadvantage of impairing reproducibility. Thus, the use of more defined media throughout the different steps is preferable. Cultured Day-7 blastocyst can be either immediately transferred to the recipient uterus or cryopreserved.

1.3.5 Achievements of In Vitro Embryo Production

Overall, substantial improvements in the IVP technique in sheep and goats have been achieved by several research groups worldwide. Importantly, the outcomes reached in small ruminants are similar to those of the bovine species, regarding both cleavage and blastocyst rates. However, few limitations still need to be overcome. The particular logistics of oocyte recovery from live females and the significantly low quality and heterogeneity of recovered oocytes are major challenges that deserve attention. Additionally, further efforts to increase IVP efficiency upon the use of defined media should be strengthened. This would increase reproducibility in IVP protocols, facilitating their acceptance and supporting a safer strategic planning regarding its implementation. Despite these limitations, the later advancements support the scenario of broader commercial demand for IVP in the goat and sheep industries in the near future.

1.4 Working with Embryos

1.4.1 Assessment of Embryonic Quality

The most reliable approach to determine quality/viability of *in vivo*- or *in vitro*-produced embryos is to check its capacity of deriving live and healthy offspring. Assisting in completing the gestational period and providing neonatal care are further approaches desirable for the ultimate success of those reproductive techniques. In most species of mammals, including small ruminants, morphological evaluation of embryos is the least invasive and most common approach used to infer the competence of embryos to develop, and to result on a term-lasted pregnancy. Currently, the International Embryo Technology Society classifies embryos into four quality categories: excellent or good (Code 1), regular (Code 2), poor (Code 3) and dead or degenerated (Code 4) (Mapletoft et al. 2020). Other more invasive techniques used to assess embryo quality include biopsy followed by evaluation of expression of genes related to embryo implantation capacity and/or quality, kinetic of development, embryo metabolism, embryo cell count and resistance to cryopreservation (Fryc et al. 2021; Brair et al. 2020; Souza-Fabjan et al. 2014, 2016).

1.4.2 Embryo Transfer

Transferring embryos to sheep and goat recipients requires those females having their estrous cycle synchronized to the developmental stage of the embryo. Estrous synchronization of recipients most often comprises the use of intravaginal devices

containing progestogens, and application of eCG one day before, or at the time of the device removal. Progestogen treatment can last for as long as 14 days (Gibbons et al. 2019, Shin et al. 2008) or shorter, such as for 6 days (Morais et al. 2020; Figueira et al. 2019). The dose of eCG normally varies from 200 to 300 IU, and in short protocols, cloprostenol (37.5 μg) might be administrated together with eCG (Morais et al. 2020; Figueira et al. 2019; Gibbons et al. 2019). Most commonly, *in vivo*-derived embryos are at the morula or blastocyst stages when recovered and transferred, thus, recipients receive embryos 7 or 8 days after device removal (Figueira et al. 2019, Gibbons et al. 2019), or 7 days after estrus onset (Fonseca et al. 2018). The synchrony of embryonic developmental stage and recipient estrous cycle day affects pregnancy rates. Cryopreserved sheep blastocysts produced higher pregnancy rates when transferred on day 8, in comparison to day 7, after device removal (Gibbons et al. 2019), and both fresh or frozen/thawed goat embryos at more advanced stages (blastocyst, expanded blastocyst and hatched blastocyst) resulted in a higher kidding rate, when compared to embryos in earlier developmental stages (morulae and early blastocysts), both groups transferred on day 7 after estrus onset (Fonseca et al. 2018). *In-vitro* derived embryos are more often cultured until the compact-morula or blastocyst stage, i.e.: for 6–8 days after IVF (Souza-Fabjan et al. 2019), and transferred to synchronized recipients on day 6 or 7 after estrus onset or 7 or 8 after device removal (Baldassarre 2021; Cox and Alfaro 2007). However, IVP embryos might be transferred on day 2 after fertilization (4-cell stage), in this case, recipients are on day 2 or 3 after estrus onset and embryo is deposited into the oviduct, instead of into the uterine horn as for embryos in more advanced stages (Baldassarre 2021). Overall, the number of transferred embryos vary from one to four according to the expected pregnancy rate, which depends on developmental stage (a higher number of 4-cell embryos are transferred), embryo source (*in vivo* or *in vitro*), preservation state (cryopreserved or fresh) and quality (Baldassarre 2021; Figueira et al. 2019; Fonseca et al. 2018).

Before embryo transfer, recipients must be checked for the presence of at least one corpus luteum, and embryos must be deposited into the uterine horn or oviduct ipsilateral to the ovary that contains this structure. Corpus luteum examination can be performed one day before transfer, by transrectal ultrasonography (Morais et al. 2020), or, immediately before the transfer, by visual inspection. The techniques for transferring embryos vary from more to less invasive ones: laparotomy, semi-laparoscopy, laparoscopy, and transcervical. The former three are surgical methods, requiring general and local anesthesia, and involves the deposition of embryos into the uterine or oviduct lumen, using a wall-penetrating small catheter. Laparotomic embryo transfer comprise visual inspection of ovaries for location of corpora lutea and exposure of a larger portion of the uterus (and maybe oviduct), for the deposition of the embryo. This technique is often used for embryo transfer to the oviduct (Baldassarre 2021; Shin et al. 2008; Cox and Alfaro 2007) and, in some cases, still used for embryo transfer to the uterine horns, but with a higher risk of promoting adhesions and compromising fertility in recipients, when compared to laparoscopic or semi-laparoscopic methods (Zohara et al. 2017; Shin et al. 2008). The semi-laparoscopic method involves observation of ovaries and location of the target

uterine horn via laparoscopy, with further external exposition of a small portion of the uterine horn via a 1–3 cm long mid abdominal incision, for the transmural application of embryos (Figueira et al. 2019; Gibbons et al. 2019; Cox and Alfaro 2007). Complete laparoscopic embryo transfer can also be performed for depositing embryos either into oviduct (Shin et al. 2008), or uterine horn (Cho et al. 2017). Transcervical embryo transfer is proposed as a least invasive technique for this purpose, not requiring complex anesthesia protocols or food deprivation, and securing general welfare of recipients (Fonseca et al. 2014). Due to marked differences in cervical anatomy between sheep and goats, transcervical approaches are easier and most commonly performed in goats (Fonseca et al. 2019a). In this latter species, no cervical relaxation is required for cervical penetration during embryo transfer. A procedure similar to artificial insemination is conducted, in order to deposit embryos in the uterine horn ipsilateral to the corpus luteum, and similar pregnancy rates to what is expected for semi-laparoscopic transfer might be achieved (Morais et al. 2020; Fonseca et al. 2014). In sheep, however, drug-induced cervical dilatation procedures are required for transposing the cervix of the great majority of individuals, but those protocols might be harmful to the corpus luteum or uterine environment of the recipient or to the transferred embryos (Fonseca et al. 2019a). Therefore, there is still a need for developing secure methodologies for non-surgical embryo transfer in sheep.

1.4.3 Embryo Cryopreservation

Embryo cryopreservation is an important supportive biotechnology for both MOET and IVP. Reduction in temperature may temporarily suspend cell metabolism, thus enabling its preservation. In this sense, cryopreservation is defined as a process for conservation of cells, tissues, or any biological constructions, by cooling them to very low temperatures (Jang et al. 2017). Specifically, embryo cryopreservation enables: optimization of reproductive biotechnologies for research and commercial purposes, preservation of surplus embryos for transfer at the appropriate time, exchange and international commerce of genetic material, conservation of genetic material from farm animals or animals under the risk of extinction, and prevention of live animal losses during transport. However, unprotected freezing is usually lethal, not due to the cells' inability to withstand storage at extremely low temperatures (below −196 °C), but because the cells are exposed to the intermediate temperature zone (−15 to −60 °C) twice: during cooling and during heating (Gao and Critser 2000; Pegg 2015). Cryogenic lesions that promote cell death are induced by osmotic stress and the formation of extra- and intracellular ice crystals. To mitigate these harmful effects, normally both, cell permeating and nonpermeating cryoprotectants are used. Protective action of permeating cryoprotectants such as dimethylsulfoxide, ethylene glycol, glycerol, methanol, butanediol and propanediol is attributed to the colligative and ligand properties with water, which reduces its intracellular cryoscopic point (Holt 2000). Conversely, nonpermeating cryoprotectants,

such as lactose, sucrose, mannitol, bovine serum albumin and polyvinyl alcohol, promote an osmotic effect, controlling the dehydration and rehydration processes and preventing the formation of large ice crystals inside the cell (Niemann 1991).

1.4.3.1 Cryopreservation Techniques

The temporary suspension of embryonic metabolism can be achieved by allowing or preventing the formation of crystals at temperatures below zero (Fig. 1.5). While the former strategy occurs at the slow freezing process, the latter is promoted by the vitrification technique. In vitrification, the high viscosity of the cryoprotectant solution (~10^{13} poises), as a consequence of the high concentration of cryoprotectants, allows the transition of water from liquid to glassy (solid) without the formation of ice crystals (Pegg 2015). The use of high concentrations of cryoprotective substances, can cause toxicity to embryos (Saragusty and Arav 2011). Conversely, in slow freezing, which requires low concentrations of cryoprotectants, embryos are less affected by cryoprotectants' harmful effects. However, those cryoprotectant concentrations are not efficient to prevent formation of ice crystals (Zeron et al. 2002). Thus, the chosen cryopreservation technique (slow freezing or vitrification) influences the types of cellular injuries (Santos et al. 2010). Regardless of the cryopreservation technique, there are numerous barriers (such as membranes and lipids) to the free diffusion of solutes, which can cause transient imbalance in the volumes

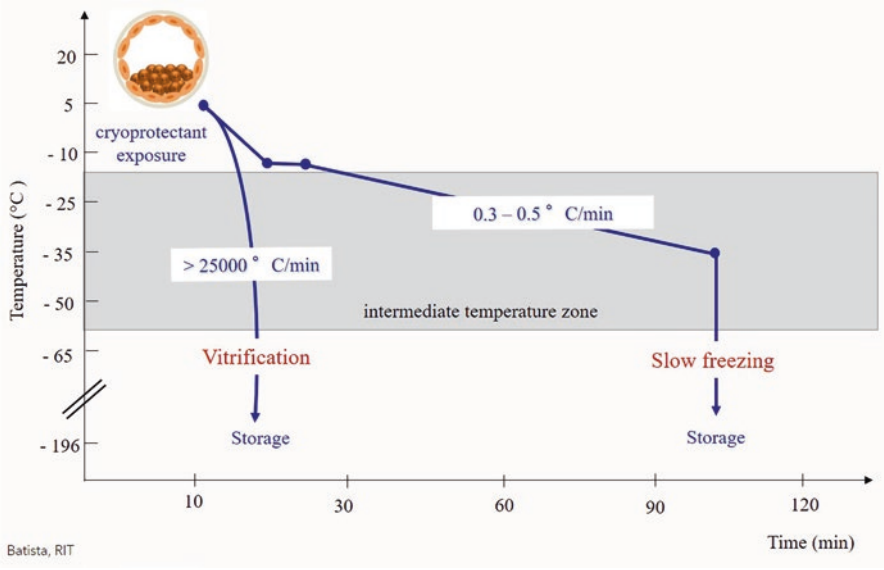

Fig. 1.5 Cooling dynamics during cryopreservation of embryos by slow freezing and vitrification techniques. The intermediate temperature zone highlights the time of embryo exposure to risk of injury by cold region of each one of the cryopreservation procedures

of the intracellular compartment, compromising cell viability (Pegg 2015). Therefore, the processes of diffusion and osmosis, which vary according to the cell type and cryoprotectant used, have important effects during the introduction and removal of cryoprotectants in the cell. These aspects are commonly subject to experimentation, and must be considered in the development of cryopreservation protocols.

Slow freezing was developed to preserve MOET-derived embryos and is widely used in commercial programs in sheep and goats. In a previous study, 1.5 M of ethylene glycol was used as a cryoprotectant, and the embryo survival rate and number of offspring born were similar for recipients that received either fresh or frozen-thawed embryos (Fonseca et al. 2018). However, slow-freezing often results in a low survival rate when applied to IVP-derived embryos (Bhat et al. 2015; Massip 2001). For this reason, vitrification methods have been studied in parallel with the development of IVP technology in different species, including small ruminants. The most used vitrification approaches for small ruminant embryos are shown in Table 1.1.

Recently, MOET-derived embryos were used to evaluate the effect of the cryopreservation technique (slow freezing *vs.* vitrification) on embryo survival rate and on expression of genes related to trophectoderm differentiation (*CDX2*), pluripotency maintenance (*NANOG*), cell proliferation (*TGFB1*), activity mitochondrial (*NRF1*) and apoptosis (*BAX* and *BCL2*) (Brair et al. 2020). The authors observed a greater change in the expression of these genes in vitrified-thawed embryos, when compared to those cryopreserved by slow-freezing or to fresh embryos. Despite this, there was no difference in the *in vitro* survival rate when the two techniques were compared (Brair et al. 2020). In general, vitrification effectiveness relies on several factors including embryonic developmental stage, origin (*in vivo* or *in vitro*), cryoprotectant solution type and volume, cooling rate, among others (Arav 2014).

A large number of ice crystals are easily formed during the slow freezing process, which results in damage to the embryo's internal structure during the cooling and reheating processes. To mitigate this effect, Li et al. (2020a) demonstrated that supplementation of *Anatolica polita*-derived antifreeze protein (ApAFP914; 10 μg/mL) to the freezing medium significantly increases the hatching rate of slow-growing sheep embryos after cryopreservation. Antifreeze proteins present in some species of plants, insects, bacteria and fungi, act as a cryoprotectant by inhibiting the formation of ice crystals during the freezing process and protecting cell membranes from damage (Mcgill et al. 2015). The *Anatolica polita* antifreeze protein acts as a cryoprotectant that binds to ice crystals, preventing the formation of large intracellular structures and reduces physical damage to cell membranes (Fletcher et al. 2001; Chakrabartty and Hew 1991). This protein also interacts directly with the cell membrane structure, which allows the embryo to adapt to low temperatures, maintaining the stability of its cell membranes' structure (Li et al. 2020a). Furthermore, minimum volume methods were proposed with the objective to reduce the volume of cryoprotectants and increase the cooling and heating rates (Yavin and Arav 2007). Using this concept, Dos Santos Neto et al. (2015) tested the effect of both Cryotop and Spatula method on the vitrification of sheep IVP embryos and

Table 1.1 Effect of the vitrification on pregnancy rate and embryonic survival

Vitrification method	Cryoprotectant	Source of the embryo	Pregnancy (%)	Embryo survival (%)	Species	References
Open pulled straws	8% EG, 18% DMSO and 0.4 M sucrose	MOET	43	14	Goat	Guignot et al. (2006)
	8% EG, 18% DMSO and 0.4 M sucrose	MOET	82	70	Goat	Yacoub et al. (2010)
	20% EG, 20% DMSO and 0.5 M	MOET	55	–	Sheep	Green et al. (2009)
	20% EG and 20% DMSO	MOET	50	35.0	Sheep	Papadopoulos et al. (2002)
	20% EG and 20% DMSO	IVP	5	2.5	Sheep	Papadopoulos et al. (2002)
	20% EG, 20% DMSO and 0.5 M sucrose	IVP	50	35.0	Goat	Ferreira-Silva et al. (2017)
10 uL plastic tips	25% G and 25% EG	MOET	64	64	Goat	Gibbons et al. (2011)
	25% G and 25% EG	MOET	50	50	Sheep	Gibbons et al. (2011)
Cryotop	15% DMSO, 15% EG and 0.5 M sucrose	MOET	66	52	Sheep	Dos Santos-Neto et al. (2017)
	15% DMSO, 15% EG and 0.5 M sucrose	IVP		39	Sheep	Dos Santos-Neto et al. (2017)
Spatula	15% EG, 15% DMSO and 60% Ficoll solution[a]	MOET	33	22	Sheep	Dos Santos-Neto et al. (2017)
	15% EG, 15% DMSO and 60% Ficoll solution[a]	IVP		11	Sheep	Dos Santos-Neto et al. (2017)
0.25 mL plastic straw	25% glycerol and 25% EG	MOET	72	35	Goat	Guignot et al. (2006)
	25% glycerol, 25% ethylene glycol and 0.4 M sucrose	IVP	21	9	Goat	Rodríguez-Dorta et al. (2007)

EG ethylene glycol, *DMSO* dimethyl sulfoxide, *MOET in vivo* derived embryo, *IVP in vitro* produced embryo

[a]Ficoll solution (0.3 g of Ficoll PM70 and 0.17 g of sucrose diluted in 1 mL of phosphate buffered saline)

observed acceptable *in vitro* survival rates for both methods. However, when the MOET- and IVP-derived embryos were transferred to recipients, embryonic survival and the birth rate were lower in Spatula, comparing to the Cryotop method (Dos Santos-Neto et al. 2017).

1.4.3.2 Effects of Cryopreservation on Embryos

Among the intrinsic factors related to the embryo that compromise its ability to survive after cryopreservation, the following can be highlighted: (i) quality, (ii) stage of development, (iii) origin and (iv) lipid content. For cryopreservation, Code 1 (excellent or good) or Code 2 (fair, Mapletoft et al. 2020) embryos are used, once embryos undergo further morphofunctional damages during cryopreservation, which normally reduce their developmental competence (Romão et al. 2015a). Regarding stage of development, a progressive increase in cryotolerance with the progression of developmental stage (4, 8 and 16 cells, morula, and blastocyst) was observed in sheep IVP embryos (Shirazi et al. 2010). A similar result was also observed when Day-2 (2–8 cells) sheep embryos were compared to Day-6 morulae and blastocysts. Despite the fact that Day-2 embryos had lower cryotolerance, the ones that survived the vitrification-heating processes had development and hatching rates similar to vitrified morulae or blastocysts (Dos Santos Neto et al. 2015). In contrast, when MOET-derived sheep embryos were vitrified, no differences in pregnancy (47% vs 50%) and embryonic survival (41% vs 50%) rates were found, when comparing morulae to blastocysts (Gibbons et al. 2011). Interestingly, although these authors reported 64% of pregnancy and survival rates when blastocysts were transferred to recipient goats, no pregnancies were obtained when morulae were transferred. As a result, in order to obtain better results regarding cryosurvival, the use of embryos in the expanded blastocyst stage, preferably of extraordinary quality is recommended.

It is well acknowledged that IVP-derived embryos have lower cryoresistance compared to MOET-derived embryos. The ultrastructural observation of fresh blastocysts showed a reduced number of microvilli, mitochondria, and mature mitochondria, as well as intercellular junctions in IVP embryos compared to MOET-derived embryos. These differences impact the resistance of embryos to cryopreservation (Romão et al. 2015a). In an attempt to improve the quality of IVP embryos, it has been shown that co-culture of IVP goat embryos with goat oviduct epithelial cells significantly improved embryo gestation and survival rates and lead to the birth of healthy offspring, after the transfer of vitrified-warmed embryos (Rodríguez-Dorta et al. 2007). In addition to the ultrastructural variations, another factor that compromises the cryosurvival of IVP embryos is the excessive presence of lipid droplets, especially in embryos cultured in the presence of serum. The detailed mechanism for this increase in lipid content in IVP embryos is still under investigation. However, some theories suggest that serum lipids can be absorbed by the blastomeres, altering the dynamics of mitochondrial β-oxidation and facilitating the assimilation of saturated fatty acids and cholesterol by cell membranes (Barceló-Fimbres and Seidel Jr 2007; Abe et al. 2002; Sata et al. 1999). Both de-lipidation during the *in vitro* development of embryos in the presence of *trans-10 cis-12* conjugated linoleic acid, and de-lipidation after associating centrifugation to a cytoskeleton stabilizer (cytochalasin D), improved the cryotolerance of IVP sheep embryos (Romão et al. 2015b). These data demonstrate that it is possible to increase embryonic cryosurvival by manipulating its lipid content.

1.4.4 Embryonic Biopsy: Gender and Diseases Diagnosis

In small ruminants, embryo sexing can be performed, and it is a promising alternative for selecting the desired gender of offspring. This might have interesting applications, such as the preferable production of females in herds interested in producing milk. Not only commercial dairy herds might be interested in adopting this technology, but especially, herds of transgenic animals that secrete proteins of interest in milk (see following sections). The current technique of choice for embryo sexing is the PCR-based detection of sequences specific to either gender (Tavares et al. 2016; Jaayid et al. 2014; Mara et al. 2004; Tominaga 2004; Aasen and Medrano 1990). The PCR is performed on embryonic cells, extracted via a biopsy procedure. Interestingly, the application of a duplex PCR analysis for both sex and scrapie resistance genotype in ovine biopsied fresh embryos was highly efficient (Dervishi et al. 2011). Scrapie is a lethal prion disease and the possibility of transferring embryos of the desired sex and presenting scrapie resistant genotypes is of utmost importance for MOET/IVP selection schemes. This tool is also valuable when working with edited embryos, as will be discussed later.

There are distinct methods to perform a biopsy on small ruminant embryos, e.g., microblade, micro-pipette, or glass needle. The blade-biopsy is the simplest and presents high efficiency. An important aspect to consider in biopsy is related to the ideal number of cells harvested from the embryo: a balance is necessary between sexing efficiency – since a minimum amount of DNA is required to reliable detect the sex – and embryo viability, i.e., if cells are excessively removed, embryo development can be impaired. Some studies recovered a higher number of blastomeres (16–30 cells, nearly one-third of the embryo: Kochhar et al. 2000), while others reported lower numbers, such as 5 cells (Mara et al. 2004) or 5–10 cells (Vilarino et al. 2018). Overall, lower numbers of cells are preferable due to the reasons above. Goat blastocysts biopsied using a specially designed holding pipette produced a 67% (8/12) pregnancy rate (El-Gayar and Holtz 2005). Indeed, although the number of transfers is not large, the literature is quite consistent in affirming that biopsy might not affect pregnancy and/or embryo viability (Vilarino et al. 2018; Tavares et al. 2016; Shi et al. 2008).

1.4.5 Transcriptomics and Embryo Production

The zygotic genome activation (ZGA) is the embryonic developmental transition event in which cell function control is transferred from the gene products provided by the mother to those synthesized from the zygotic genome. This is a pivotal process for the appropriate development of mammalian embryos (Schultz et al. 2018). Small ruminants' embryos and oocytes have been studied with the objective to understand aspects of the ZGA in mammals. Transcriptional maps were obtained form of goats' *in vivo*-derived MII oocytes and embryos at six stages of

development and two distinct transcriptomic profiles were detected: the first from the MII oocyte to the 8-cell stage, and the second from the 8-cell stage to the blastocyst (Li et al. 2020b). This demonstrates that the greatest changes in gene transcription occur between the 8 and 16-cell stages, a consequence of the ZGA. Furthermore, the single cell RNA-seq technique (scRNA seq) was used to characterize the expression profiles of messenger RNAs (mRNAs) and long non-coding RNAs (lncRNAs) during ZGA (4-cell versus 8-cell embryos) in goats, and findings indicated that the differentially expressed RNAs are involved in the retinoic acid receptor signaling pathway, as well as the signaling pathway that regulates stem cell pluripotency (Deng et al. 2018).

Transcriptomic analysis techniques have also been conducted in oocytes and embryos of small ruminants in order to address questions related to control of oxidative stress and to developmental maturity. To identify the critical functional genes and metabolic pathways involved in adaptation to oxidative stress, Wan et al. (2021) used the RNA-seq technique for a comprehensive analysis of mRNA expression patterns in goat 8-cell and blastocyst stage embryos under conditions of hypoxia and normoxia. The expression levels of zygotic genes, transcription factors and maternal genes such as *WEE2, GDF9, HSP70.1, BTG4* and *UBE2S* showed significant changes, and analysis of functional enrichment and protein-protein interaction network revealed that these differentially expressed genes were mainly involved with energy metabolism, immune stress response, cell cycle, receptor binding and signal transduction pathways (Wan et al. 2021). Using RNA-seq technology, transcriptome profiles of 1-month-old lamb oocytes after IVM were evaluated, in contrast to oocytes from large adult sheep (Ying et al. 2021). The low developmental competence of lamb embryos was mainly attributed to decreased expression of oxidative phosphorylation genes (*ATP5E, NDUFA7* and *COX6C*), thiol protease inhibitor (*CSTB*) and 26S proteosome component (*SHFM1*) and increased expression of *CUL1, MARCH7* and *TRIM17* (Ying et al. 2021). High-throughput sequencing technology was used to evaluate the set of microRNAs (miRNAs) and mRNAs differentially expressed in the ovaries of lambs and adult sheep and mRNAs related to hormone receptors, steroid hormone secretion and oocyte quality were significantly different between lambs and adults (Zhang et al. 2021).

1.4.6 Achievements of Working with Embryos

Reproductive biotechnologies in sheep and goats have supported the production of embryos, which have diverse destinations. A major output of MOET and IVF is the production of live animals, after transferring embryos to recipients. Commercial operations aiming to intensify genetic improvement are increasingly adopting embryo transfer for producing livestock. Semi-laparoscopic embryo transfer has been the method of choice. Transcervical embryo transfer, especially in ewes, is still

a challenge, due to the particular conformation of the cervix, but in goats, this technique has some appeal regarding the animal welfare standpoint. Upon transfer, fresh embryos result in higher pregnancy rates; however, cryopreservation of embryos allows the control of timing and strategic storage of them. Those advantageous features are fundamental for establishment of germplasm banking in those species, especially when considering preservation of breeds under the risk of endangerment. Although some challenges are still present in the cryopreservation of small ruminant embryos, such as cryoprotectant toxicity and higher fragility of IVP embryos, substantial improvements have been achieved in survival after cryopreservation and in the establishment of pregnancy, especially in MOET-embryos. Currently, the slow freezing method is preferably used for cryopreservation of MOET-embryos, while vitrification is used preferentially for cryopreservation of IVP embryos. Small ruminant flocks/herds, especially those of pharmaceutical interest, might also benefit from most advanced techniques involving embryos, such as embryonic biopsy for gender determination and diseases diagnosis. Although those approaches are far from being widely applied in commercial sheep and goat production systems, they have been used in research and have the potential to be used more intensively. Although a more specific apparatus or manipulation skills are required for performing biopsy, embryonic survival rates can be kept to expected levels of non-biopsied embryos. From the perspective of research advancements, sheep and goat embryos have been used extensively for diverse investigation involving transcriptomics. Studies on transcriptomics have a great variety of objectives and questions to be addresses, such as genome activation, stress survival, reproductive maturity achievement, among others. In summary, sheep and goat embryos have many outputs, form commercial applications to molecular investigations, and the potential for using more advanced technologies related with embryo technology is of increasing interest.

1.5 Cloning

In small ruminants, the first reports of laboratory development of genetically identical individuals, or cloning, involved the techniques of embryonic bipartition and nuclear transfer of embryonic cells (Willadsen 1986). Later, cloning by somatic cell nuclear transfer (SCNT) allowed the production of genetically identical copies of existing or existed animals, that served as donors of nuclei extracted from a differentiated somatic cell. This technique has become a common approach in recent years, following the interest raised by the birth of Dolly the sheep (Wilmut et al. 1997). Currently, the most relevant applications of cloning are the following: (i) understanding the biology of embryonic development, including oocyte activation, cell differentiation and proliferation requirements; (ii) studying gene functions, genome pluripotency, differentiation, reprogramming, and imprinting; (iii) prospecting alternative approaches in regenerative medicine; (iv) producing offspring of endangered species aiming their multiplication and conservation; (v) producing

selected livestock for wider propagation of elite germplasm; and (iv) producing transgenic animals bearing genetic-engineered genome. In general, SCNT is performed according to the following steps: (1) preparation of recipient cytoplasts from retrieving, selecting, and maturing oocytes; (2) isolation, characterization, *in vitro* culture, and synchronization of the somatic cell to be used as karyoplasts; (3) embryonic reconstruction itself by transferring the nucleus, fusing the karyoplast-cytoplast complexes and activating cells; (4) *in vitro* embryonic culture; (5) embryo transfer to synchronized recipients. In all these stages, there are determining factors for the success of the technique and the synchrony of all stages establishes the success of the production of offspring of viable clones.

1.5.1 Preparation and Culture of Oocytes

Most commonly, oocytes used as recipients (cytoplasts) in the SCNT procedure are derived from slaughterhouse ovaries. However, oocytes collected from live females can also be used. The female donor can also be previously stimulated with FSH. A larger number of oocytes were collected from FSH-stimulated donor, as compared to slaughterhouse-derived ovaries, in both goats (12.2 vs. 0.81 oocytes/ovary) and sheep (6.4 vs. 1.2 oocytes/ovary) (Yuan et al. 2019; Reggio et al. 2001). In sheep, oocytes from FSH-stimulated ovaries produced higher pregnancy rate than slaughterhouse oocytes (Yuan et al. 2019). Moreover, genetic background of oocyte donors can impact embryonic development. In goats, the genetic composition of recipient oocytes had an effect on *in vitro* and *in vivo* development of reconstructed embryos: the homologous genetic background of cytoplast and nuclear donor allowed superior development of reconstructed embryos (Liu et al. 2012). Regarding maturation status, oocytes at the metaphase II stage (MII) of meiosis are the most appropriate cytoplast for the SCNT procedure (Niemann and Lucas-Hahn 2012). In the laboratory, the harvested immature oocytes are matured *in vitro* for 18–24 h to reach the nuclear stage of MII. The conditions for maturation are similar to those for the IVP. Table 1.2 contains a summary of the main necessary conditions for preparation and maturation of oocytes to be used as cytoplasts.

1.5.2 Preparation and Culture of the Nuclear Donor Cell

The origin of the karyoplasts is the factor of greatest biological importance for SCNT. In sheep and goats, several sources of somatic cells had been chosen for SCNT, including cumulus cells, and fibroblasts from fetal or adult organs, such as ear and mammary gland (Yuan et al. 2019; Wen et al. 2014; Pereira et al. 2013; Choi et al. 2012; Lan et al. 2006; Keefer et al. 2001; Wilmut et al. 1997). Dolly was produced from a mammary gland-derived fibroblast, which inspired her name, as a tribute to Dolly Parton, an American singer that has voluptuous breasts. Table 1.3

Table 1.2 Preparation and *in vitro* culture of oocytes as source of cytoplasts in small ruminants

Species	Maturation medium	Environmental conditions	Maturation (%)	Authors
Goat	TCM199 with 10% goat serum, 10 μg/mL LH, 5 μg/mL FSH, and 1 μg/mL 17β-estradiol	18–22 h, 38 °C, and 5% CO_2	50 (415/833) to 68 (524/769)	Reggio et al. (2001)
Goat	TCM199 with 0.4 AU/mL pFSH, 2 μg/mL 17β-estradiol, 0.05 μg/mL sodium pyruvate, antibiotics (penicillin and streptomycin), and 10% heat-inactivated FBS	20 h, 38.5 °C, and 5% CO_2	64 (259/396)	Ohkoshi et al. (2003)
Goat	TCM199 with 0.022 mg/mL sodium pyruvate, 10,000 IU penicillin, 10,000 μg/mL streptomycin sulfate, 25 μg/mL amphotericin B, 10% FCS, 10 ng/mL EGF, 5 μg/mL FSH, 10 μg/mL LH, 1 μg/mL 17β-estradiol and 100 μM cysteamine	20 h, 38.5 °C, and 5% CO_2	83 (1046/1257)	Pereira et al. (2013)
Sheep	TCM199 with 10% estrus goat serum, 10 mM Hepes, 0.1 μg/mL 17β-estradiol, 10 μg/mL FSH, 8 μg/mL LH, 0.022 mg/mL sodium pyruvate	18 h, 38.5 °C, and 5% CO_2	77 (649/639)	Ren et al. (2014)
Sheep	TCM199 with 2.5 mM sodium pyruvate, 1 mM l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL FSH, 10 μg/mL LH, 1 μg/mL 17β-estradiol, 100 μg/mL epidermal growth factor, 0.1 mM cysteamine, and 10% sheep serum	22–24 h, 38.5 °C, and 6% CO_2	NI	Hosseini et al. (2013)
Sheep	TCM199 with 10% FCS, 10 μg/mL FSH, 20 μg/mL LH and 1 μg/mL estradiol	22–24 h, 38.5 °C, and 5% CO_2	71 (127/178) to 84 (183/217)	Yuan et al. (2019)

TCM199 Tissue Culture Medium-199, *LH* luteinizing hormone, *FSH* follicle stimulating hormone, *FCS* fetal calf serum, *FBS* fetal bovine serum, *NI* non-informed

provides a summary of the main features regarding sources and *in vitro* culture protocols for somatic cells, used to produce embryos by SCNT.

1.5.3 Handling and Micromanipulation Techniques

Small ruminant nuclear-transferred embryos can be produced by different manipulation procedures: conventional method using micromanipulators, and zona-free methods, using manual technique (or handmade cloning), as well as by the combined procedures using micromanipulators and manually (Fig. 1.6). For all techniques, the steps for obtaining cytoplasts and karyoplasts are decisive for the success in the production of reconstructed embryos. In sheep, in a comparative study of the

Table 1.3 Preparation of somatic cells as source of karyoplasts in small ruminants

Species	Source	Culture medium	Synchronization method	Authors
Goat	Cumulus cells isolated from *in vitro* matured oocytes	DMEM plus 10% FBS	0.5% FBS for 2–5 days	Guo et al. (2009)
Goat	Fetal fibroblast cells, fresh and cultured cumulus cells from *in vitro* matured oocytes	DMEM with 20% FBS and 50 μg/mL gentamicin sulphate	Contact inhibition for 3 days	Akshey et al. (2010)
Goat	Fetal fibroblasts	DMEM/F-12 medium with 10% FBS, 100 μg/mL streptomycin and 100 IU/mL penicillin	Serum deprivation	Wang et al. (2020)
Sheep	Fetal fibroblasts	D-MEM/F-12 medium with 10% FBS	NI	Fu et al. (2012)
Sheep	Adult fibroblasts	HTCM-199 plus 10% FBS	0.5% FBS for 4–6 days	Hosseini et al. (2013)
Sheep	Adult fibroblasts	DMEM/F-12 with 10% sheep serum and 1% penicillin-streptomycin	0.5% sheep serum for 4–5 days	Moulavi et al. (2013)

DMEM Dulbecco's Modified Eagle Medium, *FBS* fetal bovine serum, *DMEM/F-12* Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, HTCM199 HEPES Tissue Culture Medium-199, *NI* non-informed

efficiency of conventional and handmade cloning techniques, it was found that handmade cloning is a simpler, cost-effective and efficient approach for performing of *in vitro* production of cloned sheep embryos (Khan et al. 2018). In this study, higher morula percentage was achieved in conventional as compared to handmade cloning (44.1% vs. 30.4%), whereas blastocyst percentage obtained by handmade cloning was higher (12.5%) than conventional (5.3%), probably due to greater ooplasm volume (>100%), which occurs in handmade embryos. Therefore, the efficiency of cloned embryo production could be improved if a broader and more standardized handmade cloning technique was used.

1.5.3.1 Enucleation

In the SCNT technique, oocytes used as recipient cytoplasts must have their nuclear material removed. In many species, the nuclear content of oocytes in MII is not easily visible by light microscopy, due to the presence of cytoplasmic lipids (Czernik et al. 2019). Therefore, before the enucleation process, the oocyte is marked with Hoechst 33342, which emits blue fluorescence when exposed to ultraviolet light. In this way, the removal of nuclear material is confirmed by the absence of fluorescence in the oocyte or in the micropipette after enucleation. Enucleation is an extremely critical factor that influences the developmental ability of cloned embryos.

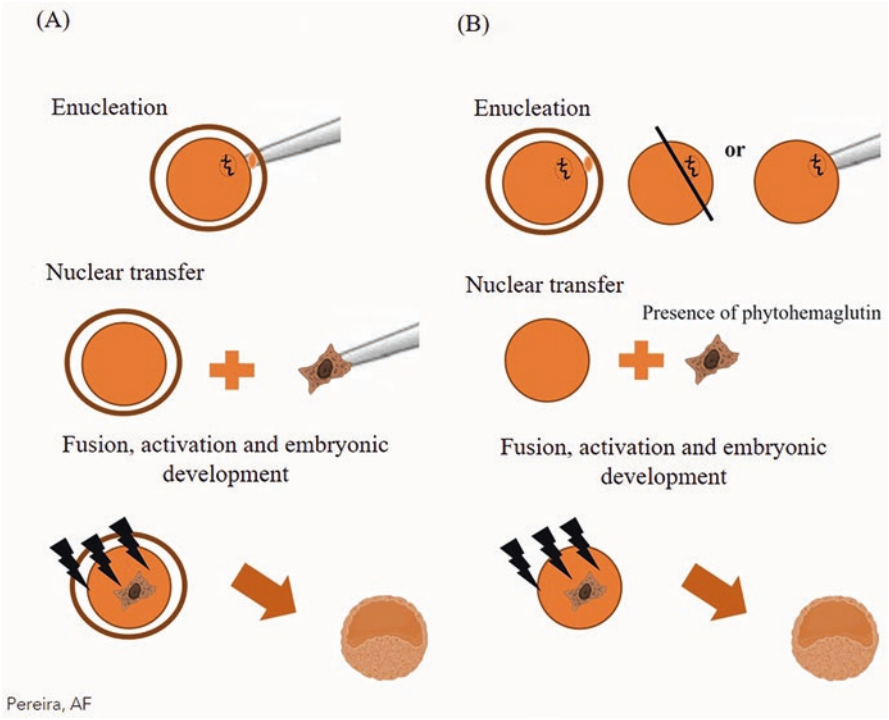

Fig. 1.6 Schematic representation of the procedures for producing clone embryos by SCNT. (**a**) Conventional Method: performed by aspiration of the metaphasic plate of oocytes in MII together with the polar body, using micropipettes coupled to micromanipulators. Donor cell individually isolated is inserted into the perivitelline space and an electrical pulse promotes the fusion of the adjacent membranes (**b**) Zona-free Methods: polar body-guided enucleation is carried out by a microblade resulting into a demi-oocyte or combined procedures using micromanipulators and manually. The donor somatic cell nucleus is sandwiched between two demi-oocytes and fused by electric current

In zona-free methods, oocytes need to undergo removal of the zona pellucid by enzymatic method before enucleation. In sheep, using both conventional and hand-made cloning, the enucleation efficiency was 93.6% and 97.0%, respectively (Khan et al. 2018). In goats, Nars-Esfhani et al. (2011) used a modified method of anaphase II/telophase II enucleation with very brief zona-free oocyte exposure to ultraviolet light, resulting in rates of 89.6% to 95.6%.

1.5.3.2 Nuclear Transfer, Fusion, and Activation

For the reconstruction of the embryo, the donor cell nucleus is transferred into a recipient cytoplasm. In the conventional method, each donor cell individually isolated based on previous culture is inserted into the perivitelline space of an enucleated oocyte for further stimulation with an electrical pulse, which promotes the

fusion of the adjacent membranes (Pereira and Freitas 2009). In the zona-free methods, karyoplasts and cytoplasts are linked by incubation with phytohemagglutinin (Pereira et al. 2013). In small ruminants, embryonic reconstruction after nuclear transfer is usually achieved by electrofusion. A significantly higher fusion efficiency was recorded by handmade cloning (96.3% vs. 92.6%) than conventional technique (Khan et al. 2018). After fusion, reconstructed embryos are subjected to artificial activation, which must mimic the role played by sperm during fertilization. Oocyte activation is an essential step in SCNT to allow subsequent development of the cloned embryos. The first somatic cell cloned goats have been produced by using electrical activation combined with cytochalasin B (Baguisi et al. 1999). Subsequently, other studies reported the use of chemical substances that include ionomycin combined with 6-dimethylaminopurine for activation of SCNT embryos to clone goats (Lan et al. 2006; Keefer et al. 2001) and sheep (Loi et al. 1998).

1.5.4 Embryonic, Fetal, and Postnatal Development

After fusion and activation, the reconstructed embryos are cultured *in vitro* until its development to the blastocyst stage. A variety of culture systems routinely used in IVP, among them, co-culture systems using primary oviduct cells or lines, are used for cloned embryo culture. In general, *in vitro* development requires supplementation of components which can assist in the development. Examples of those components are fetal bovine serum, estrous goat/sheep serum, bovine serum albumin, growth factors and vitamins, among others (Pereira and Freitas 2009; Keefer et al. 2001). However, long-term exposure to them negatively affect embryonic quality. After *in vitro* development, embryos are transferred to recipients under conditions like what occurs in embryos produced *in vitro*. Although 60-day pregnancy rates might be similar to the ones observed for IVP embryos, a low live offspring rate is observed, due to gestational lost throughout the whole gestational period (Nars-Esfhani et al. 2011). The Table 1.4 provides a summary of some results observed for to produce offspring by SCNT.

Table 1.4 *In vivo* development of embryos cloned using somatic cell nuclear transfer (SCNT) in goats and sheep

Species	Embryos transferred	Recipients	Live offspring (%)	Cloning efficiency (%)[a]	Authors
Goat	146	10	6 (60.0)	4.1	Liu et al. (2016)
Goat	500	25	18 (72.0)	3.6	Han et al. (2018)
Goat	592	37	17 (45.9)	2.9	Yang et al. (2018)
Sheep	29	13	1 (7.7)	3.4	Wilmut et al. (1997)
Sheep	139	12	3 (25.0)	2.2	Yuan et al. (2019)

[a]Cloning efficiency (%): live born clones/transferred embryos

1.5.5 Factors Affecting the Cloning Efficiency

Cloning efficiency depends on the ability of a donor cell to be reprogrammed to an embryonic stage summed up to the capability of the recipient cytoplasm to reprogram the exogenous nucleus (Ross and Feltrin 2014). Although SCNT technology has allowed several mammalian species to be cloned, abnormal embryonic development of SCNT embryos remains a main barrier to a broader and more intense use of this technology (Wang et al. 2020). Several research groups around the world have already produced embryos and live offspring. Factors related to the origin of the donor cell nucleus (karyoplast), the recipient oocyte, and protocols employed, have important effects on the *in vitro* and *in vivo* developmental capacity of the reconstructed clone embryos (Table 1.5).

The most known factors associated with unsuccessful SCNT are genetic and epigenetic errors occurring within the donor genome; incomplete genetic or epigenetic reprogramming after the nuclear transfer, or association of both reasons (Oback and Wells 2002). Serial cloning is an exceptional model for understanding the effects of genomic reprogramming errors and somatic mutations that accumulate

Table 1.5 Types of cytoplast and karyoplast and outcome in small ruminants

Species	Cytoplast (origin)	Karyoplast	Outcome	Country	Authors
Goat	MII oocytes (slaughterhouse)	Adult fibroblasts	Production of 18% and 12.7% morula and blastocysts, respectively, using transgenic karyoplasts	Brazil	Pereira et al. (2013)
Goat	MII oocytes (slaughterhouse)	MSCs and adult fibroblasts	MSCs-derived blastocysts (25.3%) higher than fibroblasts-derived embryos (20.6%)	Malaysia	Kwong et al. (2014)
Goat	MII oocytes (slaughterhouse)	Fetal fibroblasts	Blastocyst: 19.5% vs. 24.3% for manual vs. micromanipulation-based methods of SCNT	Iran	Hosseini et al. (2015)
Goat	MII oocytes (slaughterhouse)	Fetal fibroblasts	85.7% living goat rate using donor cells with overexpression of PGC7	China	Wang et al. (2020)
Sheep	MII oocytes (slaughterhouse)	Fetal fibroblasts	IP + $SrCl_2$/CB improved the quality of embryos	United Kingdom	Choi et al. (2012)
Sheep	MII oocytes (slaughterhouse)	Adult fibroblasts	12.5% blastocyst by manual cloning	India	Khan et al. (2018)
Sheep	MII oocytes (FSH-stimulated females)	Adult fibroblasts	1.5% live lambs using cytoplasts of FSH-stimulated females	China	Yuan et al. (2019)

MII oocytes those oocytes in the nuclear metaphase II stage after *in vitro* maturation, MSCs Bone marrow -derived mesenchymal stem cells, *PGC7* primordial germ cell 7, *IP + $SrCl_2$/CB* calcium ionophore + strontium chloride/cytochalasin B

gradually during development. A study developed by Yang et al. (2018) on serial cloning in goats, described the production of second-generation cloned goats from donor neonatal fibroblasts derived from first-generation clones. Nevertheless, the authors attempt to produce third-generation clones failed. The efficiency of SCNT decreased progressively with the clonal generations. A major cause of abnormal embryonic development in SCNT embryos is abnormal methylation of imprinted genes (Dean et al. 2003). Hence, restoring DNA methylation of imprinted genes is an interesting approach for enhancing SCNT efficiency. Wang et al. (2020) observed that embryos produced by SCNT are more susceptible to altered methylation patterns in imprinted genes, which is associated with their high mortality. These authors suggested that overexpression of the maternal factor Primordial Germ Cell-7 in donor cells returns the normal levels of methylation in these genes. Table 1.6 summarizes the main factors that influence cloning efficiency according to the different parameters involved in this technology.

1.5.6 Achievements and Challenges of Cloning

Cloning by SCNT has many uses on basic research, multiplication, and species conservation. The main steps of this technique are karyoplast preparation (nucleus donors), obtaining cytoplasts (recipient oocytes), and embryonic reconstruction. Extensive efforts for improving the methodologies employed on those stages have been seen in diverse lines of investigation in different species. Cloning by SCNT is increasingly being applied in small ruminants, even though its efficiency is still similar to what has been achieved since the production of the first mammal cloned by this technique. Several attempts to improve and modify the protocols for cloning by SCNT have been developed over the years, seeking to increase its efficiency.

Table 1.6 Factors that influence somatic cell nuclear transfer (SCNT) clone embryo production

Parameter	Factor
Cytoplast	Selection and oocyte maturation conditions
	Conditions of enucleation and oocyte activation
Karyoplast	Type and age of donor cell
	Manipulation techniques
	Cell cycle synchronization protocols
Embryonic reconstruction and nuclear reprogramming	Intrinsic factors, such as inappropriate or incomplete genomic reprogramming of donor nuclei
	Abnormal methylation of imprinted genes
	Epigenetic marks are cleared and re-established in the mammalian life cycle of each generation. Some epigenetic marks are retained across generations
In vivo development	Physiological problems in surviving clones
	Fetal and placental abnormalities
	Serial cloning

These attempts have been focused both on establishing appropriate conditions for obtaining karyoplasts and cytoplasts, as well as on adjusting embryonic reconstruction methods. Moreover, abnormal embryo development remains a barrier to the routine use of this technology, and mitigation of it is also being addressed. Considering the countless applications that SCNT has for reproductive and therapeutic medicine, efforts are essential to ensure greater efficiency of the technique in small ruminants.

1.6 Transgenesis

Transgenic animals and genetically modified organisms either have a segment of foreign deoxyribonucleic acid (DNA) incorporated into their genome, or an artificially induced modification in their genome sequence (Melo et al. 2007). The first successfully-reported transfer of foreign DNA to a mammal, with germ-line transmission, was obtained in mice by using a retrovirus as vector (Jaenisch et al. 1975). Ten years later, this technology was applied to farm animals as pigs, sheep, and rabbits (Hammer et al. 1985). Since the first reports on transgenic animals, a variety of applications have been suggested. In small ruminants, genetic engineering is a valuable tool for improving productive traits (milk, meat, or wool), as well as for producing disease-resistant animals. Also, sheep and goats have been used for production of recombinant proteins in milk and models to study human diseases (Menchaca et al. 2016). Great advances in animal genetic engineering were described over the past few decades. Several strategies have been used to produce transgenic small ruminants with desired traits. Additionally, improving efficiency in inducing targeted genetic modifications and simplifying the procedures were the aims that challenged scientists.

1.6.1 DNA Pronuclear Microinjection

Injection of transgenes into one pronucleus of a fertilized oocyte is the most used approach for generating transgenic mammals (Figs. 1.7 and 1.8) which has been done in a wide range of species. However, the particularities of this technique do not allow the control of all the genetic factors. The main objective of producing transgenic goats has been to obtain recombinant proteins secreted in milk. Thus, there are goats producing human tissue plasminogen activator (Ebert et al. 1991), human antithrombin (Edmunds et al. 1998), human clotting factor IX (Huang et al. 2001), spider silk (Baldassarre et al. 2003), human granulocyte colony stimulating factor (Freitas et al. 2012), lysozyme (Carneiro et al. 2018), among others. In fact, one of the only two drugs (Atryn®) available for human use is produced by transgenic goats, which secrete into their milk recombinant antithrombin III. In sheep, few studies have been published on transgenic animals secreting recombinant proteins

Fig. 1.7 Some steps of transgenesis by DNA microinjection. (**a**) insertion of media (IM) into uterine bifurcation for embryo recovery (ER) in the infundibulum. (**b**) Presumptive embryos evaluated under microscopy before microinjection. (**c**) DNA construct microinjection into one of the pronuclei. (**d**) Birth of offspring for later identification of transgenic animals. (Data related to the production of those transgenic goats has been published by Freitas et al. 2012)

in milk (Ma et al. 2017). However, the preferred target for using transgenesis in for sheep is to enhance meat or wool production (Adams et al. 2002; Damak et al. 1996).

DNA pronuclear microinjection relies upon either collecting presumptive embryos in the oviduct of superovulated donors or producing those *in vitro*. In both cases, the embryos are evaluated and those in the pronuclear stage are injected with picoliters of selected DNA into the pronucleus, using micromanipulators. The surviving embryos (~50%) are cultured *in vitro*, and later transfer to synchronized recipients, that will carry the pregnancy to term (Rülicke and Hübscher 2000). In small ruminants, prior to microinjection, embryo centrifugation is mandatory due to the difficulty in visualizing the pronuclei. Ruminant oocytes and embryonic cells have cytoplasmatic vacuoles and lipid droplets, which prevent a clear visualization of their internal organelles. Thus, centrifugation helps removing excess lipid (Pangestu et al. 1995). Centrifugation (12,100 × *g* for 5 min) of small ruminant pronuclear embryos does not affect subsequent development and facilitates the visualization and microinjection of DNA (Freitas et al. 2007). Moura et al. (2010) compared two goat breeds (Canindé vs Saanen) regarding the success of

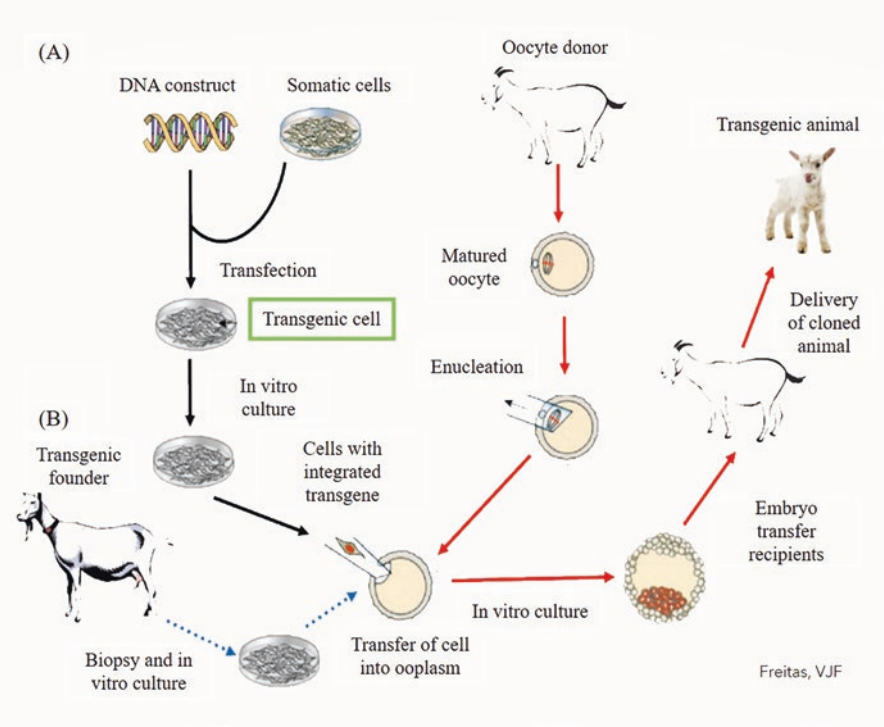

Fig. 1.8 Possibilities of obtaining transgenic small ruminants using the somatic cell nuclear transfer technique: via transfection of cell nuclei with DNA expression constructs or vectors (**a**) or by cloning transgenic founder animals (**b**). The first option is shown by black arrows, the second by blue dashed arrows and red arrows show common steps

microinjection. A higher percentage of fertilized ova was observed in Canindé (89.9%) than Saanen (36.2%) goats. In addition, Canindé donors produced a higher percentage of pronuclear embryos. Successful microinjection was observed in 96.7% of Canindé and 73.3% of Saanen embryos. The authors hypothesized that this difference was due to variations in morphometric parameters, such as zona pellucida thickness: 10.79 vs 12.63 μm, for Canindé and Saanen breed, respectively. Although DNA microinjection was the method of choice for producing transgenic models bearing gain of function modifications, the method is highly inefficient, resulting in less than 10% of the newborn carrying the targeted mutation, and with its unpredictable expression (Niemann and Kues 2007). Therefore, the random integration of genetic modifications and high incidence of mosaicism prevent the use of this method in many scientific studies or commercial projects.

1.6.2 Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) is the main route for producing transgenic livestock. Transgenic sheep and goats can be produced using SCNT via transfection of DNA expression constructs or vectors to donor cell nuclei (Fig. 1.8). Cloning transgenic founder animals is a suitable strategy for multiplying genomes of interest. Diverse cell types might work as nucleic donors to generate cloned animals. However, fetal fibroblasts have been most often used for producing transgenic cloned sheep and goats. After the production of the first transgenic cloned sheep (Schnieke et al. 1997) and goat (Baguisi et al. 1999), many transgenic and gene-targeted cloned individuals of those species have been born. The generation of transgenic animals resulting from SCNT is useful but not highly efficient. In addition, even after several improvements in technique, developmental anomalies are still frequent. Usually, less than 10% of transferred embryos result in live offspring (Menchaca et al. 2016). Abnormal epigenetic programming has been detected in clones that fail during pregnancy or have developmental impairments, and such problem must be solved as the technique is being improved (Matoba and Zhang 2018). Using SCNT method, Zhang et al. (2019) generated a transgenic goat harboring the human lactoferrin transgene. Also, embryos derived from the human α-lactalbumin-transgenic cells were transferred to recipient goats and of six female kids born, two carried human α-lactalbumin. Additionally, the protein was detected in their milk (Feng et al. 2015).

1.6.3 Lentiviral Vectors

While conventional microinjection-based methods supported the earlier production of transgenic animals, the low transgenic efficiency has encouraged the use of alternative approaches, such as lentiviral vectors. Lentiviral vectors have become an exciting new tool for developing transgenic animals due to their highly efficient incorporation into genomic DNA (Park 2007). The technique is based on microinjecting a high concentration of lentiviral construction into the perivitelline space of one or two-cell embryos, and further *in vitro* development until the blastocyst stage. This procedure allows the injection of the construction in early embryos with high efficiency and low risk of mosaicism (Menchaca et al. 2017). This technique is highly effective, as more than 95% of green fluorescent protein embryos after lentiviral microinjection and 100% of green fluorescent protein lambs were born with almost 90% showing full expression were observed (Crispo et al. 2015a). Also in sheep, high transgenesis rate was achieved by the use of lentiviral vectors combined with embryo splitting: 33.3% of blastocysts expressed green fluorescent protein and after embryo transfer, two sets of twins were born, a transgenic and a non-transgenic (Ritchie et al. 2009). Despite its good efficiency rate and lower requirement of embryo micromanipulation skills, transgenesis using lentiviral vectors has some

disadvantages, for example: small size of the vectors, random integration in multiple sites and the difficult production of high-titer virus free of contaminants (Remy et al. 2010).

1.6.4 Endonucleases

The large number of research for obtaining transgenic animals has led to the development of new techniques using endonucleases, in order to introduce modification in the DNA of nuclear donor cells, or into *in vitro*-produced zygotes. The most known ones are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated system (Cas), or simply CRISPR/Cas, and base-editing tools such as adenine base editors (ABE).

Zinc finger nucleases can induce genetic modifications via the introduction of a double-strand break into a target DNA sequence. Then, the introduction of the desired modification occurs in consequence of a subsequent DNA break repair event. ZFNs were the first available tool for targeted DNA modification. However, this technique requires reagents that are costly and laborious to be produced: factors that are discouraging its use by the scientific community. Despite no reports yet of transgenic sheep and goats produced by this technique, goat fibroblast cells were transfected with plasmids that encoded ZFN pairs, and it was demonstrated that ZFNs can edit goat β-lactoglobulin gene more efficiently (Xiong et al. 2013). Similarly, TALENs induce genetic modification by promoting a double strand break in a DNA target sequence. Further DNA break repair occurs by either the non-homologous end joining or homology-directed repair-mediated pathway, which are used to introduce the desired modification (e.g., gene disruption, gene correction or gene insertion). Proudfoot et al. (2015) used TALENs to produce myostatin genome edited sheep, and the product had a heterozygous modification at the myostatin locus. TALENs have also been used to produce knock-out goats that produced β-Lactoglobulin-free milk goat and this study was a tentative to use this species as mammary gland bioreactors for the large-scale production of human lactoferrin in milk (Cui et al. 2015).

The CRISPR/Cas system has recently been developed and recognized as an efficient tool for genome editing. CRISPR is a feature of the immune system of bacteria and archaea, and has the function to inactivate foreign nucleic acids (Horvath and Barrangou 2010). The use of CRISPR/Cas9 system, which was the first described, had undergone an unprecedented exponential increase in the last years (Doudna and Charpentier 2014). This system is easy to be explored in a molecular biology laboratory, and reagents are not as expensive as for the previous techniques and can be manufactured quickly. In sheep, CRISPR/Cas9 mRNA designed to target the myostatin (*MSTN*) locus was microinjected into *in vitro* fertilized derived zygote, producing homozygous animals of double muscle phenotype and heavier than wildtype ones (Crispo et al. 2015b). In goats, CRISPR/Cas9 system was used to generate

three *MSTN* knockout goats (Ni et al. 2014). Overall, this later technique for inducing trangenesis has significantly explored the functional role of genes related to muscle growth and body weight (such as *MSTN*) in sheep and goats, emphasizing the potential role of gene editing in the farm animal sector, with the production of individuals carrying valuable traits (Kalds et al. 2019).

Natural mutations in specific genes in the sheep have been the target of investigations that supported the current understanding on ovarian follicular physiology and the bases for gonadotropin response and prolificity in mammals (Fabre et al. 2006). Heterozygous point-mutations in specific regions in the genes *BMP15, BMPR1B* or *GDF9* (Hanrahan et al. 2004; Mulsant et al. 2001; Galloway et al. 2000) promote enhanced prolificity in sheep. These three genes are known as fecundity (Fec) genes, and are expressed in oocytes, while *BMPR-1B* is also expressed in granulosa and theca cells (Juengel et al. 2002, Wilson et al. 2001). CRISPR/Cas9 has also been successfully used to induce point-mutation in the *GDF9* gene in goats (Niu et al. 2018) and in the *BMPR1B* gene in sheep (Zhou et al. 2018). Other gene editing tools, such as ABE, based on the CRISPR/Cas9 system has been developed, with the aim to avoid double-strand breaking or the requirement for exogenous repair DNA template (Gaudelli et al. 2017). The ABE technology is used to convert a targeted single nucleotide Adenine:Thymine to Guanine:Cytosine and was also used to promote a single point mutation in *BMPR1B* in sheep with high efficiency (Zhou et al. 2020). Investigation regarding the targeted phenotype of the offspring bearing specific mutations in genes related to prolificity is still not available, once the achievements on producing live animals are quite recent (Zhou et al. 2018, 2020; Niu et al. 2018). However, those findings might represent a milestone the understanding on ovarian physiology and for improving sheep and goat industries.

The CRISPR/Cas9 system has facilitated the generation of gene-modified sheep and goats with specific milk characteristics which might also facilitate the large-scale production of useful proteins and pharmaceuticals in milk. For example, CRISPR/Cas9 system was used to remove a relevant allergenic component (beta-lactoglobulin) in goat milk (Zhou et al. 2017). This technology increases the safety of milk consumption by humans. CRISPR/Cas9 has also been used to study the gene function of stearoyl-CoA desaturase 1 in goat mammary epithelial cells (Tian et al. 2018). This gene is related to milk traits and affect the fatty acid metabolism. The use of the CRISPR/Cas9 system is now accessible to several research groups and industry due, mainly, to the easy and non-expensive molecular-based approaches required, including cytoplasmic microinjection; and to the developmental maturity of the necessary assisted reproductive technologies associated with the production of transgenic individuals (such as IVP, SCNT and cryopreservation). Moreover, a free web-based application is available to facilitate CRISPR/Cas9-mediated genome manipulation, helping with validation of target sequences, and identification of potential off-target effects (Xiao et al. 2014). It is undoubtful that CRISPR/Cas9 system revolutionized the production of bioreactor animals, driving it as an accessible technique to most laboratories and, thus, the number of research groups engaged in such technique is increasing.

1.6.5 Achievements of Transgenesis

Transgenesis has been widely applied to small ruminants. Several techniques have emerged aiming to enhance the efficiency of genetic modification and to simplify the generation of transgenic animals. These techniques include DNA pronuclear microinjection, SCNT, lentiviral vectors, and endonucleases. Currently, gene-edited small ruminants provide valuable models for research on gene functions, animal production, biopharmaceuticals, disease resistance, and hosts for the growth of human organs. Finally, CRISPR have enable the induction of single-base alterations without the need for homology-directed repair or DNA donor.

1.7 Conclusion

In this chapter, the main particularities, limitations, and latest improvements of reproductive biotechnologies involving sheep and goat females and embryo production-related techniques have been addressed. Advances in the *in vivo* embryo production involved adjusting hormonal protocols taking into consideration the female estrous cycle and wave emergency pattern, resulting in a higher number of females responding to the treatment. Progress has been achieved in hormonal-induced cervical relaxation approaches, allowed successful transcervical embryo recovery from sheep and goats. Considering the IVP approach, some recent hormonal protocols were developed in order to increase the number and quality of COCs recovered after laparoscopy in live females. For embryo cryopreservation, considerable improvements have been achieved in survival rates after cryopreservation and in the establishment of pregnancy, especially in in *vivo*-produced embryos. Techniques used for embryo transfer and cryopreservation have advanced, especially with regards to transcervical embryo transfer in goats, and vitrification of *in vitro*-derived embryos. Furthermore, conducting embryonic biopsies and performing studies with embryonic transcriptomics are promising techniques to increase de relevancy of embryo-related approaches in small ruminants.

Cloning and transgenesis are more advanced reproductive biotechnologies that have common steps to either the *in vivo or in vitro* embryo production techniques. Unfortunately, no substantial advances were observed in the last years in the SCNT cloning technique, once its efficiency is basically similar to that reported in the birth of the first animal cloned by this technique. However, considering the numerous applications that this technique has for reproductive and therapeutic medicine, further efforts are essential to ensure greater efficiency of the technique. Finally, the development of an easy and non-expensive system for generating gene edited animals by CRISPR/Cas is driving it as an accessible technique to most laboratories and the number of research groups engaged in such technique is increasing. Therefore, the majority of reproductive biotechnologies applied to the female sheep and goat have been overgoing progress throughout the last decades. Undoubtedly,

they have been impacting humankind for long. The major milestones brought by those techniques include allowing surpassing the physiological potential of a female to produce a limited number of offspring via natural reproduction; proving the possibility of de-differentiating a differentiated somatic cell so it can result in the creation of anachronic born genetically-identical individuals; and supporting the creation of biofactories (or bioreactors) consisting of females able to secrete in their milk, proteins of interest, including one medication already commercially available. From serving as egg donors, to becoming medicine makers, sheep and goat females have already made history in science and in the contemporary society.

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Chapter 2
Oogenesis and Folliculogenesis

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Abstract The ovary is a dynamic structure with two main functions. First, the ovary is responsible for producing hormones that control and direct the female reproductive system. Second, it is in the ovary that folliculogenesis and oogenesis take place. Such processes begin in prenatal life and comprise the formation, growth, and maturation of the follicular and female gamete, culminating in the release of a mature oocyte for fertilization. However, only 0.1% of the follicles reach the stage known as pre-ovulatory and are fertilized, thus underutilizing the female reproductive potential.

Here we review recent advances in oogenesis and folliculogenesis. First, we review mechanisms of formation, growth, and development of follicles. We discuss the influence of the antral follicle count in the reproductive performance in cattle. Finally, we present recent hypotheses about follicular renewal and new insights on epigenetic modifications during folliculogenesis.

Keywords Cattle · Oocyte development · Ovarian follicular population · Preantral follicles · Antral follicles · Follicular atresia · Epigenetic modifications · Neo-folliculogenesis · *Bos indicus* · *Bos taurus*

Abbreviations

AFC	Antral follicle count
AMH	Anti-mullerian hormone
bFGF	Basic fibroblast growth factor
BMP-15	Bone morphogenetic protein 15

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COC's	Cumulus oocyte complexes
DNMT	DNA methyltransferase
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GDF-9	Growth differentiation factor-9
IGF-1	Insulin-like growth factor-1
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LSD1	Lysine specific demethylase
MLL	Mixed-lineage leukaemia
MOET	Multiple ovulation embryo transfer
NSN	Non-surrounded nucleolus
OPU	Ovum pick-up
PAS	Periodic Acid-Schiff
PCNA	Proliferating cell nuclear antigen
PGF2α	Prostaglandin/D-cloprostenol
SMYD3	SET and MYND domain-containing 3
SOV	Superovulation
TAI	Timed artificial insemination
TGF-β	Transforming growth factor-β

2.1 Introduction

The ovary of mammals has two essential functions: one endocrine and the other gametogenic, the latter being responsible for the production of thousands of oocytes and follicles (Dalbies-Tran et al. 2020). Thus, the ovary, through the production of hormones, directs the female reproductive system, as well as being the place that supports the processes of folliculogenesis and oogenesis. Such processes culminate in the release of a mature oocyte for fertilization (Cox and Takov 2020). In farm animals, folliculogenesis begins at the fetal stage, when primordial germ cells migrate from the yolk sac to the primordial gonads, and then, the sequential mitotic production of germ cells occurs, establishing many groups of oogonia. Subsequently, these groups of oogonia are surrounded by somatic cells to form the cortical cords, which are the precursors of primordial follicles.

The interactions between the somatic cells of the follicle and the oocyte (i.e., communication between the granulosa and theca cells with the oocyte) are of paramount importance for the folliculogenesis process (Bernabé et al. 2020). For the

formation of primordial follicles, oogonias are differentiated, forming oocytes, in which they are surrounded by granular cells with a pavement shape. It is estimated that in cows, the population of primordial follicles is about 120,000, being reported a notable difference in the number of preantral follicles present at the birth of *Bos taurus* and *Bos indicus* females (Erickson 1966; Silva-Santos et al. 2011). The meiotic division of oocytes begins and stops at the prophase stage of meiosis I, also known as the diplotene stage. Thus, meiosis resumes only at puberty, with follicular recruitment.

After activation, the primordial follicle develops into a primary follicle characterized by an oocyte surrounded by a layer of cuboidal granulosa cells. At that time, the zona pellucida appears, a structure that covers all the oocytes present throughout the follicle development (Scaramuzzi et al. 2011). Then, the primary follicle passes to the secondary follicle stage when the granular cells multiply and form two layers of cubic morphology, in addition to the emergence of the first theca cells (Van Den Hurk et al. 2000). The hormone responsible for inducing follicle growth is called follicle-stimulating hormone (FSH). In addition, there is the appearance of follicular waves in cattle. For the follicular waves to occur, it is necessary to activate the activation of the primordial follicles. This occurs during follicular recruitment of the estrous cycle due to the contribution and increased FSH release (Padmanabhan and Cardoso 2020).

The next stage consists of the tertiary follicle. The follicle has an antral cavity filled with follicular fluid, multiple layers of granular cuboidal cells, and an entire organization of theca cells. FSH assumes an important role at this stage of development due to its action on endocrine and paracrine growth factors (Buratini et al. 2005). The presence of follicular fluid enables ultrasonographic studies, making it possible, in this way, to monitor follicular *in vivo* until its final destination: either the atresia or ovulation process. Activation of primordial follicles constantly occurs, reflecting in the pool of antral follicles that remain in growth, mainly in estrus.

In this context, the ovarian follicular population contains thousands of ovarian oocytes, most of which are included in the preantral follicles (90–95%). However, only a small portion of the ovarian follicles culminates in ovulation; the rest of these follicles undergo an apoptotic process, called atresia – a physiological event, of unknown duration (Markström et al. 2002). Thus, the quantity of primordial follicles (ovarian follicular reserve) is an important indicator of reproductive capacity, which may influence the development of reproductive biotechniques, such as cryopreservation of ovarian tissue, *in vitro* culture of follicles, OPU – Ovum pick-up (follicular aspiration), besides *in vitro* embryos production (IVEP).

This chapter covers the bases of folliculogenesis, including the most recent research findings. Discussions will also be presented on the importance of antral follicle count (AFC) as a tool in the reproductive selection of bovine females, new insights into epigenetic changes during folliculogenesis, and recent hypotheses about follicular renewal.

2.2 Oogenesis and Folliculogenesis

What actually comprises the processes of folliculogenesis and oogenesis? In this section are found the concepts established in the literature for both processes. The oocyte stock of mammalian females is formed over fetal life, through two processes: oogenesis and folliculogenesis (Collado-Fernandez et al. 2012). The physiological process of oogenesis leads to the growth and distinction of the female's primordial germ cells, which results in the fertilized haploid oocyte. On the other hand, folliculogenesis refers to the formation of follicles. It starts with the primordial follicle, and development can occur up to the stage of the mature follicle (also known as De Graaf follicle or preovulatory).

During folliculogenesis, many morphological and cytological events occur in the oocytes, which contribute toward the acquisition of developmental competence. It is these changes that enable the oocyte to progress in the process of folliculogenesis, to be subsequently fertilized, until the stage of embryo development (Walker and Biase 2020).

In the uterine environment, during fetal development, primordial germ cell are formed. These cells migrate from the yolk sac to the gonadal ridges, undergo successive mitoses giving rise to oogonia. These remain linked by cytoplasmic processes, also known as germinal cysts or nests. During this stage, somatic cells in mesonephros surround oogonias, forming a cord-like structure called cortical cords. This later form the primordial follicles. Thus, for a composition of the oocyte population that will be used throughout reproductive life, mitotic multiplication is indispensable.

There is the formation of a limited number of oocytes available for use in adulthood. The balance between the production of oogonias and apoptosis determines the availability of this range in the reproductive life of mammals (Aitken et al. 2011). After successive mitoses, in which oogonias are differentiated into oocytes, the process of meiotic division begins. There is an interruption in the diplotene stage, which belongs to the prophase stage of meiosis I. The beginning of follicular recruitment can occur at puberty or during the proximity of the end of reproductive life.

The primary or immature oocyte lasts in the stage of prophase I until just before ovulation. The meiosis process is resumed in response to FSH and luteinizing hormone (LH). Then, the other phases of meiosis occur, such as metaphase I, then anaphase I and finally telophase I. The first polar body is released and the secondary oocyte is formed. The meiotic maturation process, *in vivo*, can occur only in the oocyte of the preovulatory follicle and results, among other factors, from specific stimulation by the preovulatory peak of LH and FSH.

Furthermore, other hormonal interactions that occur during follicular development contribute to the growth of the antral follicle by mimicking or enhancing the effect of FSH on bovine granulosa cells. Among them, the main negative feedback

hormones for FSH, estradiol and inhibin, in addition to insulin-like growth factor I (IGF-1). In the metaphase II stage, there is a second interruption of meiosis. In most domestic species, the oocyte remains in metaphase II until it is ovulated and transported to the oviduct, where it can be fertilized. If fertilization occurs, the oocyte resumes and culminates in the elimination of the second polar corpuscle, thus marking the end of oogenesis. Finally, both the folliculogenesis and oogenesis process ensure a female gamete capable of being fertilized. In short, the end of folliculogenesis process happens at the time of ovulation of the mature follicle, while oogenesis ends only after fertilization.

2.3 Ovarian Follicular Population

The size of the ovarian follicle reserve (ovarian follicular population) greatly influences applied aspects of reproduction, such as fertility and pregnancy rates, thus extending the conventional concept of the ovary as a static structure. The follicular population of the ovaries may vary individually, according to species, breed, genetics, age, hormone levels, and reproductive status. Such factors can influence at quantity and distribution of ovarian follicles. It is estimated that the ovarian follicular population of bovine females, at birth, is about 235,000 follicles, ranging from 0 to 720,000 follicles per ovary (Betteridge et al. 1989).

Studies on follicular population have shown similarity in the quantity of preantral follicles in the ovaries of female zebu and taurine females. These surveys showed a follicular population for fetuses, heifers and Zebu cows, respectively, 143,929 ± 64,028; 76,851 ± 78,605 and 39,438 ± 31,017 preantral follicles. For Taurine females, the population of preantral follicles was 285,155 ± 325,195; 109,673 ± 86,078 and 89,577 ± 86,315, fetuses, heifers and cows, respectively (Silva-Santos et al. 2011).

The correlation of the population of preantral and antral follicles in the ovaries of female zebu and taurine females was also studied, in which cows with a high antral follicle count (AFC) showed a smaller amount of preantral follicles compared to animals with a low count of antral follicles. Thus, cows with low AFC had the largest population of preantral follicles (Silva-Santos et al. 2014). The authors of this study reported individual differences in the amount of preantral follicles in the ovaries of bovine females.

In relation to other species, these differ in the population of preantral follicles, such as the sheep, which has approximately 160,000 follicles, the mare about 30,000–150,000, and in women, about 2,000,000 preantral follicles. In short, this is an important field of study for understanding female reproductive physiology and assisting in the use of reproductive tools.

2.4 Ovarian Follicles

The ovary is a dynamic structure in which several events occur simultaneously, including follicular growth and development. In this section, a summary of each type of ovarian follicle will be presented, from morphological characteristics to the formation process, as well as the follicular atresia.

The follicles have an oocyte surrounded by granulosa cells, in addition to the teak cells, being considered the morpho functional unit of the ovary. The follicles have endocrine (production and release of steroid hormones and other peptides) and exocrine or gametogenic functions, presenting itself as an essential element for maintaining oocyte viability. Thus, the follicle provides an ideal environment for the growth and maturation of the immature oocyte and allows the mature oocyte to reach ovulation. Ovarian follicles are distributed in the outermost portion of the ovary, also called the cortex. However, females of the equine species have a higher concentration of follicles in the region close to the ovulatory fossa and the inner portion of the ovary (medullary region; Gonzalez et al. 2017).

In the ovarian follicles population, there are preantral or non-cavitary follicles (primordial, primary, and secondary) and antral or cavitary follicles (tertiary and pre-ovulatory; Fig. 2.1). It is suggested, for the initial phase of follicular growth, a predominantly local action, and several growth factors were identified in the first follicular changes. Among the most studied, are the Kit Ligand, bFGF (Fibroblast growth factor), LIF (Leukemia inhibitory factor), and GDF-9 (Growth differentiation factor-9).

Thus, according to the degree of follicular evolution, the ovarian population is divided into preantral or non-cavitary follicles (order of development: primordial, primary and secondary) and antral or cavitary (tertiary and pre-ovulatory follicles).

About 90% of the follicular population is a province of preantral follicles. These promote the continuous renewal of the antral follicles in the ovary (Sutton et al. 2003). However, about 99.9% of preantral follicles do not develop into preovulatory follicles and culminate in ovulation. The process that characterizes the degeneration of these follicles is called atresia. For this reason, the ovary can be considered an

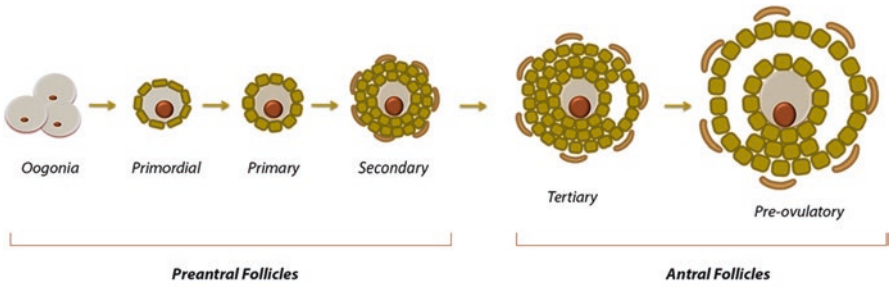

Fig. 2.1 Follicular development

organ of very low productivity since no follicle formation in adulthood, making the follicular reserve of mammalian females finite (Griffin et al. 2006).

2.4.1 Preantral Follicles

The preantral or non-cavitary follicles are differentiated by the shape and number of layers of the cells that surround the immature oocyte. The primordial, primary and secondary follicles are characterized differently according to morphology. The primordial follicles have an oocyte surrounded by a layer of 4–8 cells of the flattened or paved granulosa; the primary obtains an oocyte surrounded by a layer of 11–12 cells of the cuboid granulosa and the secondary ones have from two layers of cells of the cuboid granulosa. Also, these follicles are classified according to their integrity.

The follicles classified as intact have oocytes with less than three cytoplasmic vacuoles, germinal vesicles, the basal lamina, and intact nucleolus. Unhealthy follicles are classified as atretic, in which initial atresia (stage I: oocyte with more than three cytoplasmic vacuoles and beginning of chromatin de-densification) is considered, follicles in moderate atresia (stage II: fragmented oocyte and fragmented cytoplasm and high chromatin condensation) or follicles with marked atresia (stage III: oocyte completely fragmented or absent).

2.4.1.1 Primordial Follicles

Coinciding with the onset of meiosis, the primordial follicles are individualized from the cortical cords and are characterized by the oocyte surrounded by a single layer of granular cells in the pavement shape. In this way, the primordial follicles are formed during fetal life, in which, a layer of pavement somatic cells, known as pre-granular cells, surround the primary or immature oocyte (in prophase I), forming the first and most primitive of the follicular stages. Then, the multiplication of the pre-granulosa cells stagnates, and the primordial follicle enters the period of dormancy or quiescence. Cell proliferation is resumed only after the primordial (quiescent) follicle growth begins, in periods between months or years of its formation.

The nucleus of the oocyte occupies a central position with an evident nucleolus, and the organelles are uniformly distributed within or close to the cytoplasm. Among the organelles, mitochondria have a rounded shape, and their recognition is evident. The smooth endoplasmic reticulum and the Golgi complex are poorly developed, also with several vesicles remain scattered throughout the cytoplasm. Regarding oocytes in the ovary, most of these are stored in the primordial follicles. A high number of follicles have degenerated at this stage, and the remaining follicles will constitute the ovarian follicular pool. After establishing the follicular pool, a regular number of follicles undergo recruitment, apparently according to the

chronology of their formation. This recruitment process lasts for the entire reproductive life. The mechanisms involved in follicular recruitment and activation are not yet well established. It is suggested 100 days for the primordial bovine follicle to develop until the preovulatory stage.

2.4.1.2 Primary Follicles

The follicular recruitment is not entirely understood. It is relatively well accepted a predominance of local factors acting at the beginning of follicular growth, like Kit Ligand, GDF-9, bFGF, and LIF. Since its recruitment, the primordial follicle turns to a primary follicle whose granulosa cells have a cuboid shape present in greater quantity and more robust (Cox and Takov 2020). The oocyte seems to undergo only a necessary maturation process since its size does not change significantly. The oocyte appears to undergo only a necessary maturation process, since its size does not change significantly.

A notable change is the appearance of the pellucid zone. This structure is present separating the oocyte and cells from the granulosa. Genetic variations in zona pellucida proteins were associated with unsatisfactory results in i*n vitro* fertilization, highlighting the importance of an accurate evaluation of this structure in the oocytes. As with primordial follicles, the cytoplasm of oocytes in primary follicles also contains numerous rounded mitochondria. With the development of the follicle, the mitochondria become longer.

2.4.1.3 Secondary Follicles

The secondary follicles have a larger oocyte, a well-developed pellucid zone and the first cells of the teak are recruited and at least two layers of granulosa cells are identified. The nucleus of the oocyte passes from the central position in the oolema of the primordial follicles to an eccentric region, located in the region between the pellucid zone and the center of the oocyte. Organelles also move and stay around. The smooth endoplasmic reticulum increases in size, as most mitochondria become more elongated.

During the secondary follicle formation, some markers have been reported, as activin–A, EGF (epidermal growth factor), and BMP-15 (bone morphogenetic protein 15). At the end of this stage, the role played by the gonadotrophins can be detected, and the important actions of FSH and LH are initiated. Some markers, such as activin and its binding protein – pholistatin – have been found in primordial follicles up to the large antral follicles. The interaction of the oocyte and granulosa cells is carried out through the intercommunicating junctions (GAP junctions) in the secondary follicles and subsequent stages.

2.4.2 *Antral Follicles*

The class of antral or cavitary follicles comprises tertiary follicles and De Graaf follicles, also known as mature, preovulatory, or dominant. With the intense proliferation of granulosa cells, the follicular antrum appears, an area filled with follicular fluid, characterizing the antral follicle (Fig. 2.2). In cattle, the antral cavity can develop in follicles whose diameters vary from 0.14 to 0.28 mm. The diameter of the primordial follicles increases from 0.020 to 0.040 mm (bovine) to more than 10 mm before ovulation. Two estrous cycles are required for the follicle to grow from the beginning of antrum formation (0.13 mm) to preovulatory size.

The tertiary follicles are made up of an oocyte surrounded by the pellucid zone, several layers of granulosa cells, a small antral cavity, basal membrane, and two layers of theca cells (internal teak and external teak). They are characterized by the presence of numerous microvilli within the pellucid zone and numerous rounded and elongated lipid particles and mitochondria. A more significant number of Golgi complexes can be observed, and the cortical granules are distributed in the ooplasm, and microtubules can also be seen.

De Graaf's follicles represent the terminal stage of follicular development. There is a predominance of rounded mitochondria, and the smooth and rough endoplasmic reticulum is observed in large quantities. Cortical granules and microtubules can also be identified in the ooplasm of the oocyte. The perivitelline space is formed at this stage of development, and there is an increase in the number of vesicles and Golgi complexes. At the end of this phase, there is an indication of the marginalization of the fibrillar centers, causing the inactivation of the nucleolus function. Thus, at the same time, there is a decrease in the transcriptional activity of the oocyte.

In this context, FSH has been considered an essential factor due to its endocrine and paracrine roles. For example, FSH plays a modulation on FGF (fibroblast

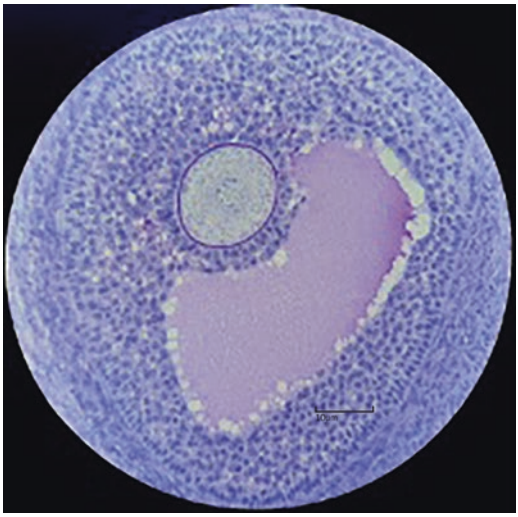

Fig. 2.2 Bovine antral follicle. Periodic Acid-Schiff (PAS) Staining. (Scale bar: 10 μm)

growth factor) family, as the FGF-8. After that occurs the antrum formation, which represents a remarkable step of follicular growth. Deviation and dominance are crucial modifications at this time, and essential aspects about LH and its receptor have been described in this stage. Furthermore, the presence of follicular fluid allows ultrasonographic evaluations, an essential method for *in vivo* studies until the final fate of the follicle: atresia or ovulation.

2.4.3 Follicular Atresia

Among all ovarian follicles, only a small portion culminates in ovulation; the rest of these follicles undergo an apoptotic process called atresia (Markström et al. 2002). Follicular atresia does not occur equally during follicular development. It refers to a physiological process of unknown duration, suggesting that it is one of the elements that controls the number of selected follicles until ovulation. The precise duration and stage of development at which the ovarian follicles culminate in atresia are not known. The atresia process differs between preantral follicles (these being the primordial, as well as the primary and secondary) and antral follicles.

For preantral follicles, the first signs indicative of atresia occurs in the oocyte, such as nuclear chromatin retraction and oocyte fragmentation, which triggers the process of irreversible elimination of ovarian follicles at this stage of development (Fig. 2.3). In the granulosa cells of the preantral follicles, alterations are rarely observed. It should be noted that after the formation of the antrum, there is a change in the sensitivity of the granulosa cells and also in the oocytes. The oocyte becomes highly resistant from this stage, and the first changes indicative of atresia are observed in the granulosa cells. The appearance of granulosa cells with pycnotic

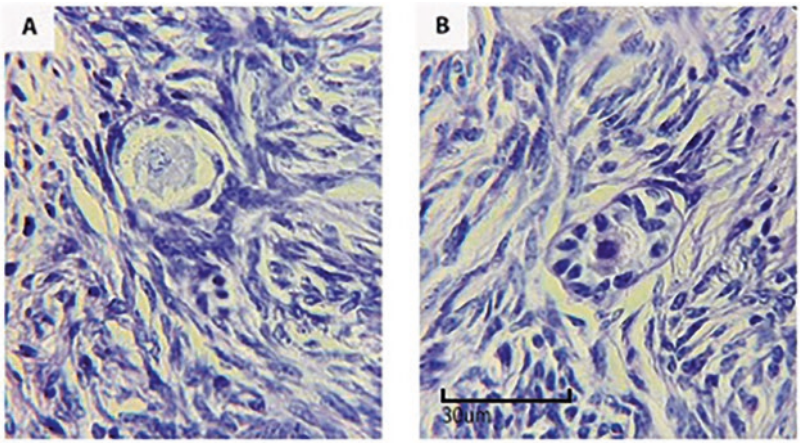

Fig. 2.3 Bovine atresic preantral follicles (**a**; **b**). Periodic Acid-Schiff (PAS) Staining. (Scale bar: 30 µm)

nuclei, chromatin condensation, and nuclear retraction can be considered the first morphological signs of atresia, which is observed predominantly in granulosa cells vicinity of the antral cavity. Subsequently, fragments of pycnotic nuclei of apoptotic bodies are observed in the antral cavity.

With the progression of atresia, there is a reduction in the number of layers belonging to the granulosa cells, as well as the invasion of the follicle by fibroblasts and also macrophages. After these drastic changes in the granular layer, the oocyte frequently undergoes pseudo-maturation, fragmented, and, finally, is eliminated during the final stages of atresia. Despite being a physiological phenomenon, atresia significantly reduces the number of potentially valuable oocytes, consequently decreasing the production of viable oocytes during an animal's reproductive life (Veitia 2020).

2.5 Antral Follicle Count

As seen in Sects. 2.4.1 and 2.4.2 of this chapter, the ovarian follicular population can be divided into preantral (these being the primordial, primary and secondary follicles) and also antral (tertiary and pre-ovulatory follicles). The population of preantral follicles represents 90% of the ovary, being responsible for the constant renewal of antral follicles. However, about 99% of the preantral follicles do not culminate in ovulation because they undergo a degenerative process called apoptosis or atresia. Thus, the ovary can be considered an organ of very low productivity.

As already described, the formation and development of ovarian follicles (folliculogenesis) begin during fetal life, and at the calving, the ovarian follicular reserve of a heifer to be used throughout the reproductive life is already established (Dalbies-Tran et al. 2020). Moreover, after the stages of primary and secondary follicular development are complete, the growing follicles give rise to antral follicles. Antral follicular development is characterized by the formation of the antral cavity, which consists of follicular fluid that was derived mainly through peripheral plasma transudation via the follicular basal membrane. The presence of the follicular fluid facilitates ultrasonographic studies that enable monitoring the follicles *in vivo* to determine their final destination, atresia, or ovulation. The stable course of these two events throughout life after birth leads to a gradual decline in the number of oocytes.

Despite the reserve of follicles in the ovary refer to a significantly larger population of antral follicles, the ovarian follicular reserve can be efficiently monitored by AFC (Ireland et al. 2008). The number of antral follicles, as well as the ovarian follicular population, refer to a high correlation represents a very strategic relationship since by using a simple ultrasound examination to determine the AFC, it is possible to estimate the female's ovarian follicular reserve. In this context, the ovarian follicular population can also be monitored efficiently using the anti-Mullerian hormone (AMH) dosage (Grigoletto et al. 2020).

AMH is produced by granulosa cells, both from antral and preantral follicles, belongs to the transforming growth factor beta (TGF-β) superfamily. Therefore, the potential of a female's ovarian reserve can be assessed indirectly by AFC, using an ultrasound examination to quantify the number of antral follicles AFC or by measuring AMH (Sakaguchi et al. 2019). Recently, Castro et al. carried out a study in which *Bos indicus-taurus* heifers (N = 68) had AFC monitored from weaning (about 9 months of age) to yearling (24 months). At the end of the year, heifers were classified as high (≥40 follicles), intermediate or medium (≥20 and ≤25 follicles) and low AFC (≤10 follicles) according to ultrasound examinations.

The AFC in the wave emergence was 47.3 ± 7.5a for the high group, 23.4 ± 2.2b for intermediate, and 8.5 ± 2.8c for the group with a low count. The correlation analysis between AMH concentration and AFC showed a coefficient of 0.80 ($P < 0.0001$), demonstrating a strong positive correlation between the two variables. This result showed that heifers with different AFC exhibit different concentrations of AMH. However, the concentration of AMH usually accompanies the number of antral follicles, revealed by the strong correlation between the variables as already well established in other studies, both in taurine and zebuine cattle.

In the following sections, the context of AFC as a reproductive trait with high repeatability, AFC and embryo production, and what has been described about AFC and fertility for Timed Artificial Insemination (TAI) will be presented.

2.5.1 Antral Follicle Count, A Reproductive Characteristic with High Repeatability

The antral follicular population is formed by tertiary and preovulatory follicles, also called the Graafian follicle. The appearance of the first tertiary follicles is observed in cattle at 230 days of gestation (Erickson 1966), wherein the presence of the antral cavity is the main structure that defines this stage of follicular development. The follicular antrum is an area filled with follicular fluid, resulting from an intense proliferation of granulosa cells. In bovine, this cavity of the antral follicles may reach a diameter of 0.14–0.28 mm, and the diameter of the follicles increases from 0.020 to 0.040 mm in primordial follicles to over 10 mm in preovulatory follicles.

Two or three estrous cycles are necessary for a follicle to develop from the initial formation of the antrum (0.13 mm) to the preovulatory stage. Moreover, it is important to emphasize that the presence of the follicular antrum is the main characteristic that allows the identification of antral follicles in the ultrasound examination. In cattle, there is a high variation among females in terms of the number of antral follicles in the ovaries, but there is high repeatability within the same individual. Repeatability values have been considerably high, ranging from 0.86 to 0.96 in *Bos taurus* females (Burns et al. 2005), 0.93 in *Bos indicus animals* (Morotti et al. 2018),

and 0.90–0.92 in *Bos indicus-taurus* females referring to weaning until the age of age (Silva-Santos et al. 2014; Morotti et al. 2017).

The breed and/or subspecies appears to have a substantial effect on this feature, which is particularly evident when comparing *Bos taurus versus Bos indicus*. However, selecting females based on their AFC (follicles with diameter ≥3 mm) during follicular waves it is possible due to the high level of repeatability of AFC found among individuals, regardless of race, as well as age, season, lactation stage or in relation to management conditions (Burns et al. 2005; Ireland et al. 2007). Normally, it is possible to determine on any day of the estrous cycle, being possible to identify with high precision the females with low, intermediate/medium or high AFCs according to the follicular waves.

For example, a study in beef cattle (*Bos indicus-taurus*, Braford – 3/8 Hereford × 5/8 Nelore, n = 137) evaluated whether different AFC populations change from the prepubertal period to the moment after puberty (Morotti et al. 2017). In this study, heifers were evaluated by ultrasonography at intervals of 60 days (Day 0, 60, 120, 180, 240, and 300) from weaning (9 months-old; Day 0) to yearling ages (24 months-old; Day 300) to verify the number of antral follicles>3 mm in diameter. Out of 137 heifers monitored, 18.3% showed AFC ≥ 41 follicles (G-high) considering all evaluations, 63.5% of the animals obtained CFA between 12 and 40 follicles (G-intermediate) and 18.3% of heifers ≤10 follicles (G-low) in all exams. The AFC showed a variation of about 21 times among women (range of 3–64 follicles) however, it was repeatable (G-high = 0.90; G-intermediate = 0.89 and G-low = 0.92) considering individuals from the same group (Fig. 2.4).

Therefore, based on this information, it is possible to select females according to their AFC, even when they are young (after weaning – prepubertal period), due to

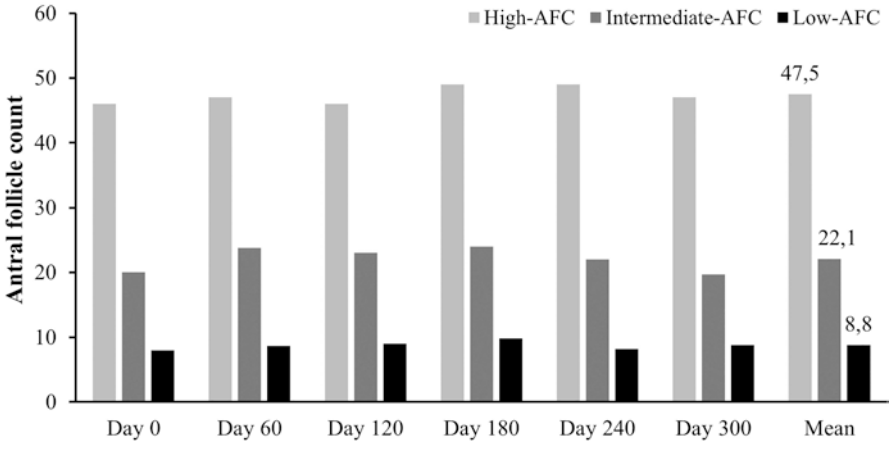

Fig. 2.4 Ultrasound assessments to determine AFC from weaning (prepubertal period) to ages of one year (after puberty) in heifers according to high- (≥ 41 follicles, n = 25), intermediate- (12 and 40 follicles, n = 87) or low-AFC (≤11 follicles, n = 25). *AFC* antral follicle count

repeatability of the observed size of the AFC at different time points. Furthermore, determining the AFC of cattle using ultrasonography is easy and inexpensive and provides a useful marker for selecting females according to the number of antral follicles, thus contributing to the positive outcome of breeding programs in the herd.

2.5.2 Antral Follicle Count and Embryo Production

It is suggested that AFC can be used as a tool to select females with better possibilities for good performance when subjected to certain reproductive biotechniques. Donors with high AFC have good fertility characteristics, as a greater number of viable oocytes and embryos produced (Ireland et al. 2007, 2008), a higher concentration of circulating progesterone, and a better pregnancy rate compared to low AFC cows (Mossa et al. 2012; Martinez et al. 2016; Vasconcelos et al. 2020). Thus, through a single ultrasound examination, it is possible to perform a better recommendation to reproduction and biotechnology in cattle (Morotti et al. 2015; Zangirolamo et al. 2018).

Since AFC can be determined only through the ultrasound exam, the observations of AFC during estrous cycles may be necessary for understanding the regulation of cattle fertility. Particularly because relatively high AFC is positively related to increased responsiveness to gonadotropin treatments during superovulation (SOV), as well as to a greater number of oocytes recovered for *in vitro* fertilization (Garcia et al. 2020), a higher percentage of oocytes normal, a more significant number of transferable embryos, in addition to higher pregnancy rates after *in vitro* fertilization (Ireland et al. 2011; Santos et al. 2016), shorter birth intervals and also greater fertility (Mossa et al. 2012).

This remarkable repeatability in the AFC per animal has enabled the use of ultrasound as a predictor of superovulation response, and the most recent research seeks to understand the relationship between antral follicle count and fertility. Thus, a simple method, ultrasonography, can be used not only to predict embryo production but also for fertility in other biotechnologies involving cattle. Besides, it was described that a high correlation of AFC with concentrations of the AMH (Grigoletto et al. 2020). This hormone is associated as an endocrine marker of superovulation response, as best oocyte-donors for *in vitro* embryo production in cattle (Ireland et al. 2011), and an indicator of herd longevity regarding fertility in cattle (Mossa et al. 2017).

In *Bos taurus*, *Bos indicus*, and *taurus* x *indicus* crosses, animals with a high plasma concentration of AMH present a greater AFC than those with a low concentration of this hormone in the bloodstream (Baruselli et al. 2016). Furthermore, in taurine females with high AFC, several physiological and endocrine peculiarities are positively associated with reproductive fertility, such as high conception rates, a short interval between deliveries, and low service rates (Evans et al. 2012). Other studies have shown that animals with higher AFC have a large amount of morphologically healthy follicles (Ireland et al. 2008).

On the other hand, low AFC cows were had inferior physiological characteristics and reproductive performance than animals classified with high AFC (Ireland et al. 2011). These females, for example, showed a low response to ovarian superstimulation treatments, low potential for embryo production (Singh et al. 2004; Ireland et al. 2007), low circulating levels of progesterone and also AMH (Martinez et al. 2016), and decreased thickness of the uterine endometrium (Jimenez-Krassel et al. 2009). Considering the production of embryos *in vitro* (IVEP) and *in vivo* (SOV/embryo transfer), the success of both reproductive biotechniques depends on the donor's AFC population. This factor is often considered a particular ovarian characteristic of the donor. It can interfere with the quantity and quality of the recovered oocytes/embryos.

In addition, other factors such as genetics, race, age and cyclicality can interfere with the success of biotechnology (Watanabe et al. 2017; Sanches et al. 2019; Ferré et al. 2020). Thus, for the production of embryos *in vitro* and *in vivo*, there may be a considerable variation in the number of embryos obtained (Ireland et al. 2007; Silva-Santos et al. 2014). Donors with high AFC exhibited a relevant number of viable embryos produced *in vivo* per animal (Center et al. 2018). Likewise, animals with high AFC showed higher embryonic production than females with low AFC when submitted to OPU/IVEP procedures (Santos et al. 2016). In Taureans, the number of *in vitro* fertilization (IVF) embryos per animal in donors with high AFC (≥25 follicles) was four times higher than in donors with low AFC (≤15 follicles).

In multiple ovulation embryo transfer – MOET, the number of embryos recovered after artificial insemination was 10.6 ± 2.7 vs. 4.7 ± 0.7 for the high and low AFC group, respectively (Ireland et al. 2007). *Bos indicus-taurus* donors with high AFC (≥40 follicles) produced more blastocysts than females in the low AFC group (≤10 follicles) for both IVF and MOET (Silva-Santos et al. 2014). Similar team-related results are less consistent for Zebu cattle. Thus, high AFC showed better performance in embryo production, however the cleavage rate was similar between low (≤7 follicles), intermediate (18–25 follicles) and high AFC (≥40 follicles; Santos et al. 2016).

Although this whole context of AFC may have a difference between works, such as management type, herd, methodological differences, and others, the main findings for embryo production, both *in vitro* and *in vivo*, show that strategically high AFC donors have a better overall performance about low AFC donors. Follow the main results of studies that evaluated the *in vitro* (Table 2.1) and *in vivo* (Table 2.2) embryos production in donors with high and low AFC.

According to a study using young embryo donors *Bos indicus-taurus*, it considered the efficiency of embryo production by OPU/IVEP and SOV among donors with high or low AFCs (Silva-Santos et al. 2014). Regarding the number of cumulus-oocyte complexes recovered, it was higher for women with high AFCs (36.9 ± 13.6 versus 5.8 ± 3.4; $P < 0.05$), resulting in a greater number of total embryos for females with high versus low AFCs (6.1 ± 4.5 versus 0.5 ± 0.8; $P < 0.05$;). In addition, the average number of embryos per collection was evaluated, in which females with high AFCs were higher than animals with low (6.9 ± 5.3 versus 1.9 ± 2.1; P < 0, 05). Thus, the level of embryo production is closely related to the size of the

Table 2.1 Performance of *in vitro* embryo production (IVEP) in *Bos taurus*, *Bos indicus* and *Bos indicus-taurus* (crossbred) cattle classified with high and low antral follicle count (AFC)

Author	Subspecies/ Breeds	AFC group	Oocytes (m ± se)	Blastocyst (m ± se)	Blastocyst rate (%)
Ireland et al. (2007)	*Bos taurus* (Beef heifers)	Low ≤15 follicles	7.5[b]	1.3[b]	29.6
		High ≥25 follicles	29.5[a]	4.9[a]	30.9
Silva-Santos et al. (2014)	*Bos indicus-taurus* (Braford)	Low ≤10 follicles	5.8 ± 3.4[b]	0.5 ± 0.8[b]	9.5
		High ≥40 follicles	36.9 ± 13.7[a]	6.1 ± 4.5[a]	16.5
Santos et al. (2016)	*Bos indicus* (Nelore)	Low ≤7 COC's	3.8 ± 1.1[b]	0.6 ± 0.6[b]	13.0
		High ≥40 COC's	40.4 ± 10.6[a]	18.4 ± 6.7[a]	41.9
Monteiro et al. (2017)	*Bos indicus* (Nelore)	Low <15 COC's	10.8 ± 0.4	3.6 ± 0.2	33.9
		High ≥15 COC's	21.2 ± 1.0	7.1 ± 0.4	34.2
Rosa et al. (2018)	*Bos indicus* (Nelore)	Low ≤31 follicles	536	203	38.6
		High ≥92 follicles	617	251	40.6
Garcia et al. (2020)	*Bos indicus* (Nelore)	Low ≤15 follicles	7.9 ± 0.6[b]	1.7 ± 0.2[b]	28.8 ± 3.9[b]
		High ≥25 follicles	30.1 ± 2.2[a]	10.3 ± 0.9[a]	41.9 ± 2.8[a]

Different lowercase letters (a–b) for the same author indicate differences (P ≤ 0.05) between the AFC groups. *AFC* antral follicle count, *COC's* cumulus oocyte complexes

Table 2.2 Performance of *in vivo* embryo production (Multiple ovulation embryo transfer) in *Bos taurus* and *Bos indicus-taurus* (crossbred) cattle classified with high and low antral follicle count

Author	Subspecies/ Breeds	AFC group	Flushes (n)	Transferable embryos/animal (m ± se)	Proportion transferable (%)
Ireland et al. (2007)	*Bos taurus* (Beef heifers)	Low ≤15 follicles	21	3.8 ± 0.8[b]	79.8[a]
		High ≥25 follicles	19	5.4 ± 1.3[a]	50.7[b]
Silva-Santos et al. (2014)	*Bos indicus-taurus* (Braford)	Low ≤10 follicles	20	1.9 ± 2.1[b]	89.5[a]
		High ≥40 follicles	20	6.9 ± 5.3[a]	78.4[a]

Different lowercase letters (a–b) for the same author indicate differences (P ≤ 0.05) between the AFC groups. *AFC* antral follicle count

population of antral follicles in the donors; moreover, it showed that cows with high AFCs performed better in embryo production *in vitro* and *in vivo*. However, it revealed interestingly that in donors with high AFC it does not seem to affect the embryo production technique, but in donors with low AFC, the MOET technique is more efficient.

Most studies suggest that high AFC can generate better reproductive performance for embryo production, both *in vitro* and *in vivo*, compared to low AFC, the proportion of embryos remains with some doubt. The general numerical values for embryonic production are higher in a high AFC donor who has a numerical advantage that repairs minor losses during the process. However, the fact that embryonic rates are similar between low and high AFC donors is still not entirely clear. In IVEP, for example, there is a donor with high AFC that presents high oocyte recovery but converts little embryos, and its blastocyst rate is low. In this context, according to the studies, there was no distinction in the blastocyst rate between the low and high AFC group (Santos et al. 2016; Rosa et al. 2018). On the other hand, MOET donors with high AFC tend to have high embryo production and a high embryo recovery rate (Ireland et al. 2007; Silva-Santos et al. 2014).

Regarding the proportion of transferable embryos, Silva-Santos et al. (2014) reported no difference for females with high AFCs compared to low (78.4% versus 89.5%). In contrast, Ireland et al. (2007) described a smaller proportion of embryos transferred to females with a greater number of antral follicles (50.7% versus 79.8%) after SOV. These authors suggested that the smallest number of transferable embryos in the group with the high AFCs was due to lower oocyte quality, possibly due to the lower concentration of circulating FSH found during the follicular waves. These findings provide important information because, in addition to the effect of the AFC, the ovarian response appears to be influenced by gonadotropin-stimulated FSH release or a by the relationship between AFC and FSH.

In addition, it is important to consider the development phase in which the follicles are aspirated, since there is direct interference in oocyte competence for IVEP (Seneda et al. 2020). Cavalieri et al. (2018) studied cows submitted to follicular wave synchronization, in which they found a better rate of IVEP and pregnancy rate after embryo transfer considering the females aspirated on a random day of the estrous cycle. Thus, oocyte recovery for animals with different AFC must be considered. To this end, a study evaluated the effect of synchronization regarding follicular emergence before OPU/IVEP in embryo donors with low and high AFC (Garcia et al. 2020). This study revealed that high AFC donors respond better to total oocytes, viable oocytes, number of embryos, and pregnancies compared to low AFC donors, as well as the use of synchronization resulted in improvements in the number of embryos and pregnancies compared to donors in which the OPU was carried out at random.

However, the most interesting is that this study revealed an interaction between AFC and synchronization, showing that the donor with high AFC synchronized responds better to most of the IVEP variables and number of pregnancies. This information is very important because it represents another recommendation for donor selection, first of all, genetic merit must be considered. High AFC donors can

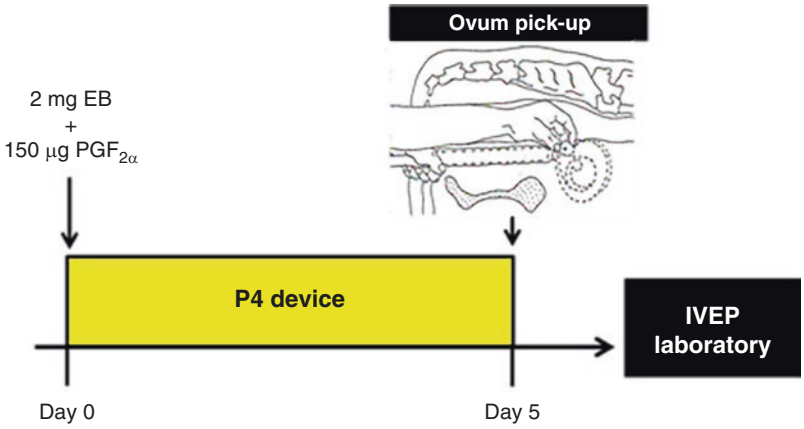

Fig. 2.5 Hormonal treatment protocol to control the emergence of the follicular growth wave before the ovum pick-up procedures and *in vitro* embryo production. *EB* estradiol benzoate, *IVEP in vitro* embryo production, *PGF2α* prostaglandin/D-cloprostenol, *P4* progesterone

be selected (Morotti et al. 2017), and finally, to be more efficient in the *in vitro* technique, a program for synchronizing the wave emergence before the OPU can be employed. Although wave emergence can be synchronized with a series of hormonal protocols that are effective, Garcia et al. (2020) initiated the hormonal protocol on any day of the estrous cycle (Day 0) with an intravaginal progesterone device and intramuscular administration of 2 mg of estradiol benzoate and 530 μg of D-cloprostenol. Then, on day 5, the device was removed and after follicular aspiration (Fig. 2.5).

2.5.3 *Antral Follicle Count and Fertility to Timed Artificial Insemination*

If, on the one hand, efficiency in the production of embryos is positively related to the number of antral follicles, the relationship between AFC and reproductive performance at timed artificial insemination (TAI) is still controversial. Several studies reported a high conception rate after using TAI in low AFC females of the subspecies *Bos indicus* compared to high AFC animals (Moraes et al. 2019; Lima et al. 2020). In addition, according to Jimenez-Krassel et al. (2017), they found that Zebu females with high AFC had lower fertility and shortened reproductive life compared to females with low AFC. Therefore, the whole context that links AFC and fertility to artificial insemination is not yet fully established, mainly because initial studies in *Bos taurus* cattle reported that females with low AFC were associated with several characteristics of low fertility (Ireland et al. 2011; Evans et al. 2012; Mossa et al. 2012).

Table 2.3 Conception rate and number of antral follicles in Nelore cattle with different antral follicle count following a timed artificial insemination program

Author	AFC groups	AFC (m ± sd)	Conception rate % (n/N)
Morotti et al. (2018)	Low (≤15 follicles)	11.3 ± 2.8^{c}	61.7^{a} (150/243)
	Intermediate (20–40 follicles)	29.4 ± 6.0^{b}	52.9^{b} (210/397)
	High (≥45 follicles)	52.8 ± 7.7^{a}	49.5^{b} (96/194)
	P-value	0.001	0.02
Moraes et al. (2019)	Low (≤10 follicles)	7.7 ± 2.6^{c}	57.7^{a} (176/305)
	Intermediate (11–29 follicles)	18.0 ± 3.4^{b}	49.7^{b} (155/312)
	High (≥30 follicles)	38.0 ± 12.4^{a}	47.9^{b} (57/119)
	P-value	<0.0001	0.008
Lima et al. (2020)	Very low (≤15 follicles)	10.6 ± 0.2^{d}	57.9^{a} (374/646)
	Low (16–30 follicles)	23.9 ± 0.2^{c}	53.1^{a} (214/403)
	Intermediate (31–44 follicles)	37.3 ± 0.3^{b}	54.9^{a} (116/211)
	High (≥45 follicles)	53.1 ± 0.6^{a}	45.2^{b} (76/168)
	P-value	<0.0001	0.001

Different superscripted letters in the same column and author indicate differences ($P \leq 0.05$) in the AFC (a–d) and conception rates (a–b) among the AFC groups. *AFC* antral follicle count

In this context, the dynamics of the follicles in the ovaries and the pregnancy rate were evaluated in Nelore cows with low and high AFC submitted to TAI programs (Morotti et al. 2018). This study demonstrated that animals with low AFC had higher follicular diameters and pregnancy rates than those with high counts (Table 2.3). Corroborating these findings, cell proliferation in follicles in the final stage of development – indicating a more intense follicular activity in cows (*Bos indicus*) – was considered higher in females with low AFC than in high AFC (Machado et al. 2017). Other studies have also revealed an interaction of AFC with the body condition score in *Bos indicus* cattle (Moraes et al. 2019) and longer reproductive life and greater reproductive performance in bulls with low AFC (Jimenez-Krassel et al. 2017).

Gene expression was regulated positively in oocytes and cumulus cells of Nelore females with low AFC, which also showed a higher concentration of progesterone and greater fertility to TAI. These genes act on intercellular communication, meiotic control, epigenetic modulation, cell division, follicular growth, cell maintenance, steroidogenesis and response to cell stress (Lima et al. 2020). Because of the context discussed so far, it is notable that AFC has been used as a selection tool for donors to OPU/IVF procedures and MOET due to the quantitative advantages to embryo production in females with high AFC. However, a major concern is regarding the future fertility of herds if a massive sealing is carried out, according to the AFC. Everything indicates high AFC seems to be more advantageous for embryo production, while low AFC seems to be more strategic for artificial insemination.

This reproductive characteristic has low to average heritability in cattle, considering a positive aspect. There was also a low correlation between genetic merit and the number of antral follicles. And yet, there is no link between AFC and milk

production, considering the lactation of Holstein cows (Walsh et al. 2014). For Braford cattle, the parameters related to zootechnical production and genetic selection showed a low correlation with AFC (Morotti et al. 2017). AFC can be considered a selection tool, in which it does not cause genetic demerits for the progeny. And it can be used as a secondary strategy for the selection of donors, reconciling the evaluation of genetic merit (Morotti et al. 2017).

Finally, several aspects need to be further investigated concerning AFC. For example: In assisted reproductive biotechnology, can hormonal needs vary depending on AFC? What is the real impact that nutrition and health can have on AFC in females' reproductive age? Is AFC linked to any molecular, gene expression, or other mechanisms that can better explain fertility differences? Another unknown point is whether the mothers 'AFC and fathers' testicular characteristics can impact their daughter's AFC. In summary, several studies have tried to verify the influence and/or the correlation of the AFC in the reproductive performance in cattle. So far, it is not possible to define the full role of AFC in global fertility, due to the controversial results, even from the same team. It is quite predictable that some specific aspects may be related to reproductive efficiency. For example, it is clear the use of AFC for *in vitro* and *in vivo* embryo production; however, we do need a better understanding of the matter for TAI and fertility.

2.6 Epigenetics and New Concepts – Neo-Folliculogenesis

Information and studies on oogenesis and folliculogenesis have increased significantly in recent years. Several aspects are related to this growth. In the commercial view, the industry of embryo production has increased worldwide, mainly for the *in vitro* production of cattle embryos. Also, TAI and timed embryo transfer have been widely used due to the benefits of genetic improvement. Considering basic research, some topics of follicular physiology remain unknown (Gershon and Dekel 2020). For example, the *in vitro* culture of preantral follicles has emerged as a useful model, but this technique's efficiency still needs some improvement.

Moreover, new hypotheses have been described about the population of follicles, generating debates regarding oogenesis, folliculogenesis, as well as about the mechanisms of follicular substitution. Finally, new areas of science, like epigenetics, bring interesting perspectives for increasing our understanding of ovarian physiology. This section discusses the new hypothesis on the continuing production of oocytes. Finally, in the following topics, a brief review of epigenetic alterations during the folliculogenesis process will be presented, and discussions on the follicles with more than one oocyte (multi-oocytes), bringing to light the concept of neo-folliculogenesis.

2.6.1 Continued Oocyte Production?

As already described in the previous items, the finite and non-renewable supply of ovarian germ cells is considered as a basic premise of the physiology of reproduction. The gradual postnatal decline of the number of oocytes occurs mainly due to apoptotic mechanisms, and the absence of ovarian germ cells is a widely accepted phenomenon in the senile age. Although this concept was described in 1870 for Waldeyer and it has been accepted for more than 150 years, Johnson and collaborators (2004, 2005) have presented evidences to consider the revision of this topic. These authors have described the occurrence of oogenesis and folliculogenesis after birth. Stem cells would be considered the origin for that (Albamonte et al. 2019).

Stem cells have great importance in the current scientific scenario (Yu et al. 2020). Due to particular characteristics, as self-replication and differentiation in other specialized cells, stem cells are closely involved in cellular and genomic therapy; both themes are considered important promises in biomedical research. The stem cells have different levels of plasticity. The meaning of plasticity refers to the ability of a stem cell to generate cells from different tissues, no matter from which embryonic layer it is originated.

For instance, it has been demonstrated that bone marrow stem cells can differentiate into neural cells, myocytes, hepatocytes, and cardiac cells. The demonstration of stem cell plasticity can be considered a suggestive argument in favor of the neo-oogenesis hypothesis (Bhartiya and Sharma 2020). However, many criticisms have been considered against the theory of neo folliculogenesis. Important questions have been emerged. Regarding clinical aspects, the occurrence of the ovarian inactivity in senile females is a widely accepted phenomenon.

2.6.2 Epigenetics and Folliculogenesis

Epigenetics refers to the study of heritable phenotypic changes that do not involve alterations in the DNA sequence. In other words, it means the information carried by the genome that is not coded by DNA. In eukaryotes, chromatin is the state in which DNA is packaged within the cell, basically organized in a group of proteins comprising the core (H2A, H2B, H3, and H4) linker (H1) histone. The fundamental unit of chromatin is the nucleosome, in which it is formed by an octamer of two units from each of the four central histones surrounded by 147 base pairs of DNA.

The important and dynamic role of the nucleosome to control gene expression has been reported. Covalent changes, as well as the interaction between histones and DNA, can vary, allowing for significant changes in DNA transcription. These covalent modifications involve the addition or removal of chemicals groups that modify DNA and chromatin cohesion. Acetylation, methylation, and phosphorylation represent some of these chemical reactions. These modifications are

predominantly reversible, providing a dynamic mechanism for activating or repressing DNA transcription (Fig. 2.6).

Proteins are the final product of DNA transcription, and they represent the agents of physiological activity. Although the DNA sequence is stable in an organism, the transcription is modulated according to epigenetic mechanisms. For example, if a nucleosome drives a lower attraction between chromatin and DNA, transcription will be facilitated, and the result will be an increase of the protein coded in that DNA sequence.

On the other hand, a tight contact between DNA and chromatin will cause an inhibition of the transcriptional activity. We can consider epigenetics as a bridge connecting the genome with the phenotype. Epigenetics also plays an essential role in the evolutionary process because it can modulate gene expression accordingly to external signals. This aspect is particularly important in germ cells once genetic information undergoes generation by generation.

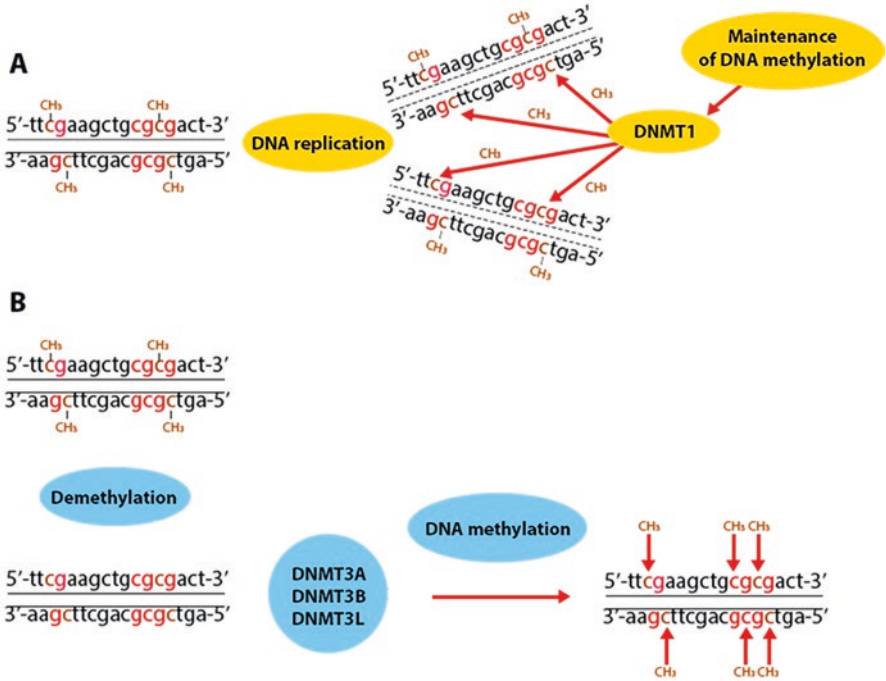

Fig. 2.6 Types of strategies for DNA methylation. (**a**) Mechanism of methylation maintenance carried out by DNA methyltransferase 1. (After DNA replication, the newly synthesized DNA strands (dashed lines) gain methylation only in cytosines of 5′-CpG-3′ sites that are paired with methylated CpG sites on the parent strands (solid lines). Thus, the DNA methylation pattern that existed before replication is reestablished). (**b**) Mechanism of de novo methylation carried out by DNMT3A, DNMT3B, and DNMT3L. Enzymes can recognize unmethylated DNA and establish a new methylation pattern using a replication-independent mechanism. *DNMT1* DNA methyltransferase 1, *DNMT3A* DNA methyltransferase 3A, *DNMT3B* DNA methyltransferase 3B, *DNMT3L* DNA methyltransferase 3L

As the epigenetic concepts are relatively new, their importance in regulating physiological mechanisms is currently being investigated to gain a better comprehension of this field. On folliculogenesis, research findings on epigenetics are revealing interesting information. For growth and differentiation of the oocyte, epigenetic impressions are established in the form of DNA methylation. In addition, epigenetic mechanisms play additional regulatory roles, acting on large-scale changes in the structure of chromatin and in the control of gene expression. Moreover, the oocyte represents an interesting model for studying the DNA methylation process since the entire methylation panel is established from scratch in a cell that does not divide (Demond and Kelsey 2020).

In the various species of mammals, there are interactive changes in the structure and function of chromatin, which occurs during the development of the oocyte. The changes in the chromatin structure are characterized by the initially de-condensed configuration called the non-encircled nucleolus (NSN). There is a progression of growth and differentiation, the oocytes undergo a change in the nuclear organization in which the chromatin becomes progressively condensed, forming a ring of heterochromatin around the nucleolus, a configuration called the surrounded nucleolus (SN). Most oocytes have the SN configuration, in the germinal vesicle stage. And there are still changes in the levels of transcription; oocytes that show the NSN configuration suffer from high transcription rates, while those in the SN state have a shortage of transcription.

Methylation of histone H3-K4 is considered a specific process of the dynamic gene performed by histone lysine methyltransferases such as Meisetz, MLL (mixed lineage leukemia) and SMYD3 (containing 3 SET and MYND domains). Histone H3-K4 demethylases such as LSD1 (specific lysine demethylase) indicated a highly regulated coordination between methylation states to control gene expression in response to hormonal stimuli, such as androgen receptor activation. Research on paracrine and endocrine regulation of oogenesis and folliculogenesis indicated specific regulatory pathways for oocytes and granulosa for meiosis and mitosis. H3K4 and LSD1 play a central role in regulating the transcription of germ cells and support cells during spermatogenesis and oogenesis.

The hormones estrogen and FSH stimulate the activity of Aurora-B kinase in the ovary. They regulate the proliferation of granulosa cells. And the phosphorylation of histone H3-S10 was detected in granulosa cells in proliferation of developing follicles, in the primary to antral stages. In contrast, H3-K4 methylation in granulosa cells was highly restricted. For all the H3-K4 methyl brands examined, little or no reactivity was detected in the granulosa cells from the primary to the tertiary stage. It was noted that a positive regulation of H3-K4 methylation occurred in the granular cells of the antral follicle, and strong signals were detected in the innermost granular wall and in the cumulus. The emergence of H3-K4 methylation in the antral follicles occurred synchronously with the functional and morphological changes as the preantral granules separate into populations that form cumulus and the granular wall.

There was a decrease in the H3-K4 methylation signals in the granulosa cells according to the distance from the oocyte. Cells with H3-K4 methylation in antral

follicles may have a specialized granular population, coordinating signaling between the oocyte and the somatic cells. It is suggested that in the antral follicles, the oocyte controls the behavior of the granulosa. This may be due to the production of TGF-β signaling molecules, called BMP-15 and GDF-9. In the granulosa cell, BMP-15 and GDF-9 co-expresses with the aim of FSH responsiveness and granulosa proliferation. The temporal appearance of H3-K4 methylation in the granulosa manifests a potential link to TGF-β signaling and maturation, in which it is dependent on FSH for the preovulatory stage. Upstream regulators are elucidated and the gene targets for H3-K4 methylation in the mural and in the granulosa cumulus remain elucidated.

For instance, phosphorylation of serine 10 in the histone H3 has been correlated to increased transcription and favoring cellular division. Interestingly, this covalent modification was involved with FSH and estradiol during the preovulatory peak, showing a close interaction of this chromatin alteration and a regulatory role in follicular growth.

Regarding the effect of H3K4 on oogenesis and folliculogenesis of mammals, some results suggest an intense activity in the oocytes, from primordial follicles to large antral ones. In granulosa and theca cells, different patterns were identified, according to the number of methylations.

Mono and di-methylation are restricted to antral follicles, and H3K4 trimethylated was identified earlier in the primary and secondary follicles, suggesting a possibly local regulation during the phase. LSD1 has not been identified during follicular growth, probably because of a large activity of H3K4 during all follicular stages. Interestingly, epigenetics plays an especial role in stem cell pluripotency. H3K4 is mainly involved in the reestablishment of pluripotency of somatic cells. This scenario opens a wide field of opportunities for scientific investigation. Thus, it also suggests that epigenetics might be the starting key to understand folliculogenesis regulation and testing the new hypothesis of follicular renewal.

2.6.3 Multi-oocyte Follicles in Adult Mammalian Ovaries

Most follicles are expected to find only one oocyte. However, more than two oocytes can occur in the same follicle. These follicles are found in fetal ovaries, however the presence in adult ovaries is misunderstood. The studied aspects of these follicles in adult females, such as origin, destination, contribution in relation to ovulation and fertility, are discussed below. Follicular formation and development are already well established in most mammal species. In addition, the typical changes and the time when it occurs are described differently between species, however the general conserved pattern is considered.

At the genital crest, germ cells differentiate into oogonias and thus form nests. Oogonias undergo mitosis and interact with somatic cells to give rise to germline cysts. In cysts, oogonias are linked by intercellular bridges. There is an organization of germ cells and pre-granulosa epithelial cells in cysts, characterizing the ovigerous or ovarian cords. These remain until primordial follicles are formed. For an

Table 2.4 Frequency of multi-oocytes follicles in four mammalian species

Specie	Category	Frequency % (observed/total)	Reference
Goat	Prepubertal Non-pregnant Pregnant	100 (6/6) 50 (3/6) 17 (1/6)	Lucci et al. (1999)
Bitch	Prepubertal Mature	68.4 (13/19) 36.6 (48/131)	Payan-Carreira and Pires (2008)
Pig	Gilts Sows	6.36 (49/819) 1.38 (9/659)	Stankiewicz et al. (2009)
Bovine	Fetus Heifer Cow	40 (8/20) 37.5 (9/24) 45 (9/20)	Silva-Santos et al. (2011)

oogonia to become an oocyte, meiosis must occur, interrupted in the diplotene stage of meiotic prophase I. As for the formation of primordial follicles, the individualization of the oocytes first occurs. For this, the germline cyst degenerates. Thus, most ovarian follicles are expected to obtain a single oocyte. However, there are reports of follicles with more than oocytes inside.

It is called multi-oocyte follicles, those follicles with two or more oocytes are contained in a single follicle, in which there is no basement membrane between them. These structures are described in the following animals: bats, mice, rabbits, cats, dogs, pigs, sheep, goats, cattle and humans (Table 2.4). These follicles have already been considered as pathological changes, although some authors consider them as polymorphism due to numerical combinations of oocytes and pre-granular cells. The number of oocytes present in the multi-oocyte follicle can vary from about 2 to 24. These multi-oocyte follicles are larger in volume, about 2–3 times than follicles with a single oocyte. Multi-oocytes may be elongated or round in shape.

According to research, the presence of multi-oocyte follicles in the ovaries is not uncommon. These were found in all stages of folliculogenesis, considering the primordial stage up to the preovulatory stage. In the ovaries of young females, it was noticed a higher frequency in the first stages of growth and a lower frequency according to follicular development. Furthermore, the correlation between the variation in the number of antral follicles among heifers with the presence of multi-oocyte follicles is suggested. It was found that in follicles that contain two oocytes, they tend to be at the same stage of development. This fact is supported due to the similarity in the measurement of the thickness of the pellucid zone and the diameter of the oocyte. This relationship has not been proven in follicles with more than three oocytes (Oliveira et al. 2017).

Some research has reported that multi-oocyte follicles can culminate in ovulation. Other studies suggest that degeneration of the smallest of the follicle oocytes occurs, which may interfere with the others. In addition, atresia is likely to be the destination of atypical follicles, as is the case for most mammalian ovarian follicles. Although there is evidence indicating that multi-oocyte follicles can reach maturity,

they are not yet complete (Gaytan et al. 2014). There is no information on the selection of oocytes that survive or those selected as nutrient cells destined for degeneration. Still, it is suggested that the presence of multi-oocyte follicles in the ovaries of adult females, indicates the inability of these follicles to ovulate. This fact may justify the decrease in the number of multi-oocytes found over time (Silva-Santos et al. 2011).

Insect studies show that germline cysts are important for the development of a single oocyte through the presence of nutrient cells. However, regarding mammals there is no information. These are described at the beginning of folliculogenesis, moreover it can develop *in vitro* up to the blastocyst stage. Are they able to reach ovulation and release a viable oocyte *in vivo*, or does their presence indicate the inability of these follicles to ovulate? Further research on the function of multi-oocyte follicles and their correlation with fertility is necessary for an explanation on the subject.

2.7 Conclusion

As described in this chapter, regulation and all aspects of folliculogenesis remain a universe to be studied and explored. The extraordinary progress of the last few years is notable, in which many researches contribute so that the gaps are filled, especially in the preantral phase. How does the recruitment start? How are controlled the activation and repression of so many genes? Why are only a few follicles submitted to the growth factors per time? Is there only one central regulatory mechanism for everything? In this context, what is the real role of the antral follicle count – AFC, which can monitor the ovarian follicular reserve, as a reproductive selection tool in cattle? Due to the controversial results obtained, including from studies by the same research group, it is not possible to define the complete influence of AFC on global fertility. The use of AFC for the production of embryos *in vitro* and *in vivo* seems to be evident; however, further investigations need to be carried out for TAI and fertility.

Neo-folliculogenesis can still be considered as an undergoing hypothesis. However, it seems reasonable to predict some modifications in the present concept of the ovarian follicular pool. Perhaps this hypothesis will be partially proved or perhaps differently, but it is reasonable to believe that something new is coming. If an analogy can be made with the renewal process of neural cells, which was considered impossible some years ago, nowadays it is a well-documented mechanism. It is quite evident that epigenetics is emerging as a wide field of possibilities to study regulatory mechanisms and gene functions in the ovary and deeply investigates the hypothesis of follicular renewal. Despite the challenge, since the interaction between chromatin and DNA is a very dynamic process, recent studies indicated that the nucleosome is orchestrating a crucial role in the essential structure of the beginning of life: the ovarian follicle.

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Chapter 3
Proteomics of Animal Viruses

Suchismita Behera and Amol Ratnakar Suryawanshi

Abstract Viruses are obligate parasites, and therefore are dependent upon host's cellular machinery for their survival. Alterations in several cellular pathways occur due to viral infections where viral proteins share contributions in host-cell interactions. The study of these altered proteins and their role in viral infection is crucial to understand a biological system or disease progression, to identify markers for diagnosis and prognosis, and to develop therapeutic strategies to control the viral infections. In recent years, advancements in proteomics technologies and approaches have created an opportunity to study alterations in host/viral proteins due to viral infections in high throughput manner.

In this review, we first describe proteomics technologies and approaches. We review viral proteomics studies on common animal viruses, categorized as animal viruses, human viruses and zoonotic viruses, to understand the role of proteins altered during viral infection. This review also provides a list of proteins altered during virus infections and their probable role in viral pathogenesis.

Keywords Proteomics · Animal viruses · Zoonotic viruses · Proteomics approaches · Gel-based · Label-free · Mass spectrometry · Viral proteins · Altered proteins · Host proteins

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Abbreviations

^{13}C	Carbon-13
^{15}N	Nitrogen-15
^{2}H	Hydrogen-2 or Deuterium
1D-PAGE	One-dimensional polyacrylamide gel electrophoresis
2D-DIGE	Two-dimensional differential gel electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AHA	Azidohomoalanine
AMPK	Adenosine monophosphate-activated protein kinase
Annexin A2	Annexin 2
AP-LC-MS/MS	Affinity purification liquid chromatography-tandem mass spectrometry
Apo A-I	Apolipoprotein A-I
AQUA	Absolute Quantification
Arl3	ADP-ribosylation factor-like protein 3
ARSs	Aminocyl-tRNA synthetases
ATP	Adenosine triphosphate
BHK-21	Baby hamster kidney 21
BHV-1	Bovine herpesvirus 1
BONCAT	Bioorthogonal noncanonical amino acid tagging
Caco-2	Cancer-coli 2
CAMK2	Calcium-calmodulin protein kinase II
CAMKIIA	Calcium-calmodulin protein kinase II A
CD4	Cluster of differentiation 4
CDK1	Cyclin-dependent kinase 1
CDK7	Cyclin-dependent kinase 7
CFR	Curved-field reflectron
CHIKF	Chikungunya fever
CHIKV	Chikungunya virus
CI	Chemical Ionization
CRM1	Chromosomal maintenance 1
CRMP2	Collapsing response mediator protein 2
CRP	C-reactive protein
CSFV	Classical swine fever virus
Cy	Cyanine
DDX1	DEAD box helicase 1
DDX17	DEAD box helicase 17
DDX3	DEAD box helicase 3
DDX3X	DEAD-box helicase 3 X-linked
DEAD	Aspartate-glutamate-alanine-aspartate
DENV	Dengue virus
DNA	Deoxyribonucleic acid
DNAH12	Dynein heavy chain 12

DNAH5	Dynein heavy chain 5
DNAJC7	DnaJ homology subfamily C member 7
dUTPases	deoxyuridine-triphosphatases
EBOV	Ebola virus
EBV	Epstein-Barr virus
EI	Electron Impact ionization
ENO1	Alpha enolase
ERK/MAPK	Extracellular-signal-regulated kinase/ mitogen activated protein kinase
ESCRT	Endosomal sorting complex required for transport
ESI	Electrospray Ionization
F9	Coagulation factor IX
FABP3	Fatty acid-binding protein 3
FGL1	Fibrinogen-like protein 1
FMDV	Foot-and-mouth disease virus
FTIR	Fourier transform ion cyclotron resonance
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI2	Guanosine diphosphate dissociation inhibitor alpha 2
Ge-LC-MS/MS	Gel-enhanced liquid chromatography-mass spectrometry
GFAP	Glial fibrillary acidic protein
GLUL	Glutamate ammonia ligase
GOPC	Golgi-associated PDZ and coiled-coil motif-containing protein
GRSF1	G-rich sequence factor 1
GS	Glutamine synthetase
GTPase	Guanosine triphosphatases
HaCaT	Human keratinocyte cells
HCMV	Human cytomegalovirus
HFFFs	Human fetal foreskin fibroblasts
HFFs	Human foreskin fibroblasts
HFLS	Human fibroblast-like synoviocytes
HIF-1	Hypoxia-inducible factor 1
HIV-1	Human immunodeficiency virus type 1
HNRNPH1	Heterogenous nuclear ribonucleoprotein H1
HSP90AA1	Heat shock protein 90 AA1
HSP90AB1	Heat shock protein 90 AB1
HSV-1	Herpes simplex virus 1
Huh7	Hepatocyte-derived cellular carcinoma
ICAT	Isotope-coded affinity tag
ICP0	Infected-cell protein 0
ICP22	Infected-cell protein 22
ICP4	Infected-cell protein 4
IFITM3	Interferon-induced transmembrane protein 3
IFV	Influenza virus
IPG	Immobilized pH gradient

IRF9	Interferon regulatory factor 9
iTRAQ	Isobaric tag for Relative and Absolute Quantitation
JAK1	Janus kinase 1
JEV	Japanese encephalitis virus
LC-MS	Liquid chromatography-mass spectrometer
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LMP1	Latent membrane protein 1
LMP2A	Latent membrane protein 2A
L-particles	Light particles
LRG1	Leucine-rich alpha-2-glycoprotein 1
LTQ	Linear trap quadrupole
m/z	mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionization
MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometer
MALDI-TOF/MS	Matrix-assisted laser desorption ionization-Time-of-Flight/ Mass Spectrometer
MALDI-TOF/TOF	Matrix-assisted laser desorption ionization-Time-of-Flight/ Time-of-Flight
MCMV	Murine cytomegalovirus
MDBK	Madin-darby bovine kidney
MDCK	Madin-darby canine kidney
MDMs	Monocyte-derived macrophages
MEFs	Mouse embryonic fibroblasts
mePROD	Multiplexed enhanced protein dynamics
MFAP4	Microfibril-associated glycoprotein 4
MHV68	Murine gammaherpesvirus 68
MHVs	Murine herpesviruses
MIB-MS	Multiplexed kinase inhibitor bead-mass spectrometry
MIF	Macrophage migration inhibitory factor
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MYO18A	Myosin XVIII A
nanoLC-MS/MS	Nanoscale liquid chromatography and tandem mass spectrometry
NEDD4	Neural precursor cells expressed developmentally down-regulated protein 4
NHS	N-hydroxy succinimide
NKRF	Nuclear factor-kappa-B repressing factor
NSFL1C	NSFL1 cofactor 47
OIE	World Organization for Animal Health
ORF	Open reading frame
PBMCs	Peripheral Blood Mononuclear Cells
PCV	Porcine circovirus
PDC6IP	Programmed cell death 6 interacting protein

PDGFRα	Platelet-derived growth factor receptor α
PDGFRβ	Platelet-derived growth factor receptor β
PI3KC	Phosphatidylinositol 3-kinase catalytic subunit type 3
PI4K2B	Phosphatidylinositol 4-kinase type 2 beta
PK-15	Porcine Kidney 15
PLAT	Tissue-type plasminogen activator
PRM	Parallel Reaction Monitoring
PRV	Pseudorabies virus
PRV-Bartha	Pseudo rabies virus
bartha	
Prx-1	Peroxiredoxin-1
PSD	Post-source decay
PSMB1	Proteasome subunit beta type-1
PSMB8	Proteasome subunit beta type-8
PSMD2	26S proteasome non-ATPase regulatory subunit 2
Q1	Quadrupole 1
Q2	Quadrupole 2
Q3	Quadrupole 3
qTOF/MS	Quadrupole time-of-flight mass spectrometer
RAB8B	Ras-related protein Rab-8B
RABV	Rabies virus
Ras	GAP SH3
	Ras-guanosine triphosphatase accelerating protein Src homology 3
RBP4	Retinol-binding protein 4
Rev	Regulator of viral protein expression
RNA	Ribonucleic acid
RNH1	Ribonuclease/angiogenin inhibitor 1
RPL18	Ribosomal protein L18
RPL3	Ribosomal protein L3
RPL5	Ribososmal protein L5
RPS6	Ribosomal protein S6
RRE	Rev Response Element
SAA2	Serum amyloid A2
SAMD9	Sterile alpha motif domain-containing protein 9
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SDS	Sodium-dodecyl sulphate
SILAC	Stable Isotope Labeling with Amino Acids in Cell Culture
siRNA	Small interference RNA
STAT2	Signal transducer and activator of transcription 2
SWATH	Sequential Window Acquisition of all Theoretical Mass Spectra
TANK	TRAF family member-associated nuclear factor-kappa-B
Tat	Trans-activator of transcription

TGFBR1	Transforming growth factor beta receptor 1
TGFBR2	Transforming growth factor beta receptor 2
TLR2	Toll-like receptor 2
TMT	Tandem Mass Tag
TNF	Tumor necrosis factor
TOF	Time of flight
TOF/TOF	Time-of-flight/time-of-flight
TQMS	Triple-quadrupole-mass spectrometer
TRAF3	Tumor necrosis factor receptor-associated factor 3
TUBA1C	Tubulin alpha-1C chain
U_L	Unique long region
US22	Unique short region 22
Us3	Unique short region 3
VAMP8	Vesicle-associated membrane protein 8
VCAM1	Vascular cell adhesion molecule-1
VLPs	VP40-containing virus-like particles
VPS13D	Vacuolar protein sorting-associated protein 13D
VPS24	Vacuolar protein sorting-associated protein 24
VPS28	Vacuolar protein sorting-associated protein 28
VPS37	Vacuolar protein sorting-associated protein 37
VPS4A	Vacuolar protein sorting-associated protein 4A

3.1 Introduction

Proteome is defined as the total population of proteins of a cell, tissue, organ or organism at a particular time. “Proteome” term was first coined by Prof. Marc R. Wilkins in 1994 (Noordin and Othman 2013). Proteome has come in limelight after the completion of human genome which has estimated to have 30,000–40,000 genes in it. These genes potentially should encode similar numbers of proteins. However, alternative RNA splicing and post-translational modifications are the major factors that increase the number to more than 2,00,000 proteins from these genes (Kosak and Groudine 2004). Hence, the proteome of an organism is more complex than the genome. Changes in proteome such as modifications, translocations, synthesis or degradation occur in response to either internal or external stimuli. Therefore, the number of proteins present in a proteome can vary from a number of genes present in that genome-based on environment (Graves and Haystead 2002).

Proteomics is a high throughput approach for studying complete proteome of a biological system at a given time point (Chandramouli and Qian 2009). The term “proteomics” was first coined at Macquarie University with the establishment of first dedicated proteomics laboratory in 1995 (Anderson and Anderson 1996). The suffix “omics” is mostly used to describe big and refer to overall functioning of living system (Pomastowski and Buszewski 2014; Westermeier and Naven 2003). Proteomics aims to identify and characterize total proteins, and focuses on a more

global and integrated view of biology with a study of all proteins of a biological system (Graves and Haystead 2002). Although achievements in genomics research have provided a better understanding of the biological system, the knowledge is still incomplete without proteomics.

Proteomics covers several applications in biological research deciphering the involvement of proteins in several biological processes as well as in any altered cellular mechanisms. The ease of proteomics application in diverse research areas has emphasized the importance to resolve the complexity of biological machinery (Dominguez et al. 2007; Lottspeich 2009). Proteomics has played a major role in identifying potential diagnostic and therapeutic targets during pathophysiological conditions. The requirement of advanced analytical technology, selectivity and sensitivity landed up emergence of high-resolution proteomics techniques. Proteomics helps in identification and validation of potential biomarkers in high throughput manner. It also helps to identify the molecules involved in disease pathogenesis and understand their role in disease mechanisms to facilitate the development of vaccines (Pomastowski and Buszewski 2014). The combination of different proteomic-based methodologies and advancements in proteomic analytical platforms have expanded the role of proteomics in clinical applications (Wiktorowicz and Brasier 2016).

3.2 Mass Spectrometry-Based Proteomics

Mass spectrometry has become an integral part of proteomics which has evolved from time to time achieving improvement in technology. The major steps involved in mass spectrometry-based proteomics are the separation of proteins by various techniques and then either identification or both identification and quantification of proteins by mass spectrometer. Timeline with the invention of different proteomics technologies and approaches are shown in Fig. 3.1 and different proteomics approaches along with details of the discovery are shown in Table 3.1.

3.2.1 Separation of Proteins

In many cases, sample complexity is one of the major limitations for identifying more proteins by mass spectrometer. Hence to reduce the protein sample complexity, different protein separation techniques are commonly used. In gel-based separation technique, one- dimensional polyacrylamide gel electrophoresis (1D-PAGE) separates proteins on basis of molecular weight (Laemmli 1970) whereas two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separates proteins first by isoelectric point and then molecular weight (Görg et al. 1985; O'Farrell 1975). However, non-gel-based protein separation is mostly performed by liquid chromatography. In liquid chromatography, separation of proteins is based on various

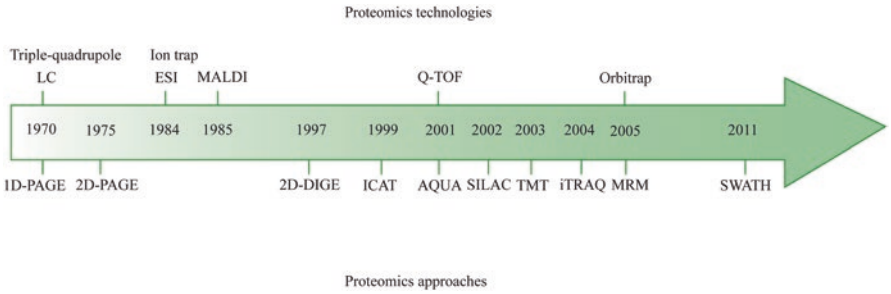

Fig. 3.1 Timeline showing emerging proteomics technologies (top) and proteomics approaches (bottom) with gradual advancement. The proteomics technologies shown in this figure include triple quadrupole, liquid chromatography (LC), Ion trap, Electrospray ionization (ESI), Matrix-Assisted Laser Desorption and Ionization (MALDI), Quandrupole-Time-of-flight (Q-TOF) and orbitrap. The proteomics approaches include one-dimensional polyacrylamide gel electrophoresis (1D-PAGE), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), two-dimensional differential gel electrophoresis (2D-DIGE), Isotope-coded affinity tag (ICAT), Absolute Quantification (AQUA), Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC), Tandem Mass Tag (TMT), Isobaric tag for Relative and Absolute Quantitation (iTRAQ), Multiple Reaction Monitoring (MRM) and Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH)

Table 3.1 Different proteomics approaches along with details of their discovery

Serial no.	Name of proteomics approach	Common name	Year of discovery	Discovered by	References
1	One-Dimensional Gel Electrophoresis	1D-PAGE	1970	Ulrich K. Laemmli	Laemmli (1970)
2	Two-Dimensional Gel Electrophoresis	2D-PAGE	1975	P. O'Farrell	O'Farrell (1975)
3	Two-Dimensional Differential Gel Electrophoresis	2D-DIGE	1997	M. Unlü	Unlü et al. (1997)
4	Isotope-Coded Affinity Tag	ICAT	1999	R. Aebersold	Bottari et al. (2004)
5	Stable Isotope Labeling with Amino acids in Cell culture	SILAC	2002	S.E. Ong	Ong et al. (2002)
6	Tandem Mass Tag	TMT	2003	A. Thompson	Thompson et al. (2003)
7	Isobaric Tag for Relative and Absolute Quantitation	iTRAQ	2004	P.L. Ross	Ross et al. (2004)
8	Absolute Quantification	AQUA	2001	S.P. Gygi	Stemmann et al. (2001)
9	Multiple Reaction Monitoring	MRM	2005	R.D. Unwin	Unwin et al. (2005)
10	Sequential Window Acquisition of all Theoretical Mass Spectra	SWATH	2011	G.L. Andrews	Andrews et al. (2011)

columns that differ according to physicochemical properties such as non-porous or porous, chemical properties like ligands with their density, geometry like diameter and pore volume or particle size and shape and composition of the column such as silica, polymers or carbon (Žuvela et al. 2019). Fractionation is one of the separation techniques where proteins can be separated either by charge through strong cation exchange (Edelmann 2011) or by polarity through C^{18} column (Agilent 2017). Fractionation of membrane proteins and several organelle proteins is also possible by various standardized protocols. Affinity chromatography is another fractionation technique particularly used to separate high-abundant plasma proteins. Removal of high-abundant proteins expands the range of analysis and identification of more proteins becomes easier (Mrozinski et al. 2008). Overall, all these separation methods help in reduction of protein sample complexity.

3.2.2 Protein Identification and Quantification by Mass Spectrometry

3.2.2.1 Overview of Mass Spectrometry

In proteomics, mass spectrometer is an instrument used for protein analysis. Mass determination and protein identification is carried out by mass spectrometry technology. The mass spectrometer was invented by J.J Thomson in 1902 (Griffiths 2009). A mass spectrometer is composed of three major components namely an ion source, an analyzer and a detector as shown in Fig. 3.2. An ion source of a mass spectrometer can produce ions that are propelled towards the analyzer by an electric field. Ions are resolved by analyzer according to mass-to-charge (m/z) ratio. These ions are then detected by the detector through a measurable signal for analysis (Cho 2007; Parker et al. 2010). An ion source of a mass spectrometer is mainly used for the ionization of molecules and commonly used ion sources are Electron impact ionization, Chemical ionization, Electrospray ionization and Matrix-Assisted Laser

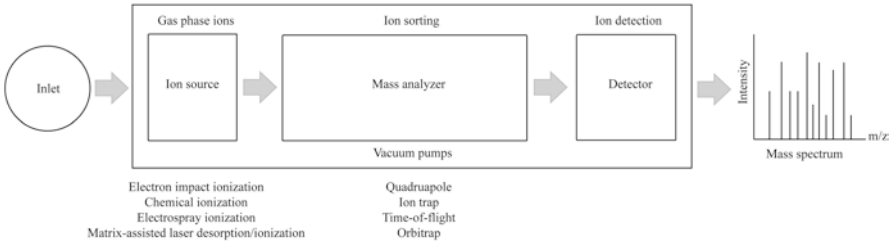

Fig. 3.2 Diagrammatic representation of basic components of a mass spectrometer include an ion source, a mass analyzer and a detector. Inlet is known for sample injection that undergoes ionization by an ion source, then the ions are sorted in mass analyzer and detected by a detector in form of the mass spectrum. Different types of ion sources and analyzers are listed at the bottom of respective components

Desorption Ionization. Similarly, quadrupole, ion trap, orbitrap and time-of-flight are various types of analyzers used in mass spectrometers.

3.2.2.2 Ion Source

Few commonly used ion sources of the mass spectrometer are described below.

Electron Impact Ionization

The ion source of a mass spectrometer is based on an ionization technique used in it. Electron impact ionization (EI) is the first ionisation method and used in a conventional instrument where sample molecules are ionized by bombardment with electrons in a gas phase at a low pressure (10^{-5} torr). Electrons energy is utilized for energy transfer to sample molecules leading to extensive fragmentation. Inside the ion source such electron beam from a filament knocks off one electron from the sample molecule. This process results in fragmentation and production of positively charged molecular ions (M^+). This ionization technique is based upon low pressure source and is considered a high energy process (Oksman et al. 1994; Parker et al. 2010).

Chemical Ionization

F.H. Field and M.S.B. Munson discovered chemical ionization (CI) for mass spectrometer in 1966 (Harrison 1992). Chemical ionization is a soft ionization technique in which ion/molecular reaction occurs within reagent gas ions and sample molecules. This process helps to achieve ionization of sample molecules.

Electrospray Ionization

Electrospray ionization technique (ESI) is an important discovery in mass spectrometry which was awarded John Fenn with Nobel Prize in 2002 (Whitehouse et al. 1985). Today, electrospray ionization is a widely used ionization method in the analysis of biomolecules and biomolecular complexes. Electrospray ionization is a technique to dissipate a liquid sample in a homogenous form of droplets. Sir Geoffrey Taylor described the underlying physical effect of the electrospray ionization technique (Taylor and A 1964). Electrospray ionization technique uses a needle at high voltage to pass sample solution. Passing of liquid sample through the needle results in a spray of small charged droplets containing the sample. After droplet formation, evaporation of solvent begins while they are in flight. Gradually, solvent molecules leave droplets as neutral particles with an increase in field density at the droplet surface (Grimm and Beauchamp 2010). The extremely high charge density

of threshold field leads to columbic explosion in less than a few microseconds and produces highly charged, stable small droplets (Gomez and Tang 2013; Grimm and Beauchamp 2010; Hager et al. 1994). Through time, several small droplets are generated and serve as a major source of ions detectable by a mass spectrometer. Generated ions bear multiple charges that are transformed into a simple mass spectrum for detection of the molecular mass of fragments (Cho 2007).

Nano-electrospray is the most efficient electrospray source with 100% ionization efficiency and generates a population of very small charged droplets (Wilm and Mann 1994, 1996). Mass spectrometer separates ions produced as charged droplets based on the m/z ratio. Thus, analysis of higher-molecular weight compounds can be performed by electrospray ionization technique as it will lead to producing highly charged particles with an apparent low molecular m/z (Parker et al. 2010). Electrospray ionization technique deals with low chemical specificity and generation of very stable ions. Moreover, high ionization efficiency contributes to the widespread distribution of electrospray ionization technique (Wilm and Mann 1996).

Matrix-Assisted Laser Desorption Ionization

In 1985, two scientists from Germany, Franz Hillenkamp and Michael Karas established the matrix-assisted laser desorption ionization (MALDI) technique (Nie et al. 2014). In a breakthrough in 1987, Koichi Tanaka along with his colleagues exhibited successful ionization of bigger molecules using nitrogen laser. Tanaka's discovery leads him to share the Chemistry Nobel Prize of 2002 for developing soft desorption ionization method for mass spectrometric analysis of biological molecules (Huang 2002). With subsequent improvisation of the technique, the first matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS) was commercially available during the early 1990s (Hillenkamp and Karas 2000).

Matrix-assisted laser desorption ionization requires sample analysis by mixing samples with an organic compound known as matrix. Irradiation of laser beam upon the mixture of sample and matrix leads to desorption and then ionization of sample analyte. Successful ionization of the sample depends upon the considerable absorption coefficient of matrix with a specific wavelength. Upon laser irradiation, the matrix absorbs a majority of photon energy to soft ionize sample analyte and generate single protonated ions (Jurinke and Oeth 2004; Singhal et al. 2015; Wu and Odom 1998). The protonated ions drift through a field-free path and separate based on the m/z ratio. Detection and measurement of charged analytes are dependent upon various kind of mass analyzers (Jurinke and Oeth 2004; Singhal et al. 2015).

3.2.2.3 Analyzers

Various types of analyzers are used in mass spectrometer which varies based on resolution, sensitivity and specificity of mass selection. Commonly used analyzers are quadrupole, ion trap, time-of-flight and orbitrap.

Quadrupole Analyzer

In addition to ionization techniques, mass analyzers are also important in the revolution of biological mass spectrometry. Because of its simplicity, quadrupole mass spectrometers are most commonly used. The presence of four parallel rods in a quadrupole mass analyzer makes it possible for specific m/z ions to pass through the detector (Parker et al. 2010).

Ion Trap Analyzer

In the 1950s, an ion trap mass analyzer was patented by Paul and Steinwedel as an alternative to a quadrupole mass analyzer. Despite similar mass filtering principles, the physical design of an ion trap mass analyzer was different from the quadrupole mass analyzer with the presence of only three electrodes. Entrance and exit electrodes allow a selective flow of ions to and from the mass analyzer whereas the ring electrode resent between them trap ions on its path. This kind of physical design in the ion trap analyzer acts as a three-dimensional quadrupole (Parker et al. 2010).

Time-of-Flight Analyzer

Time-of-flight (TOF) determines the length of time taken for the charged ion of the analyte to reach the detector in a field-free vacuum. The m/z ratio is calculated by TOF where lighter ions reach the detector earlier than the heavier ions (Parker et al. 2010).

Before the discovery of MALDI, TOF has already invented in late 1950s. Due to poor resolution, TOF took a longer time for applications in a mass spectrometer. TOF is defined as a technique where charged ions are identified based on the time required to reach and get detected by a detector (Cornish and Cotter 1993a).

Orbitrap Analyzer

In 2005, Alexander Makarov has discovered another ion trap mass analyzer known as orbitrap (Hu et al. 2005). Orbitrap consists of two outer electrodes and a central electrode where ions are trapped via electrodynamic squeezing. Once ions are trapped, oscillations of ions occur around the central electrode at different frequencies and lead to separation. After measurement of oscillation frequencies of ions, their mass spectra are acquired using image current detection (Makarov et al. 2006a, b).

3.2.2.4 Detector

The detector used in mass spectrometers detects the molecules and provides information about the m/z ratio of particular ions. These ion masses are further used to search against theoretically generated peptide/fragment ion masses of proteins present in database to identify the protein.

3.2.3 Combinations of Commonly Used Mass Spectrometer

3.2.3.1 Liquid Chromatography-Tandem Mass Spectrometer

Liquid chromatography-tandem mass spectrometer (LC-MS/MS) is a combined technique for separation of ions by liquid chromatography and mass analysis by a mass spectrometer. V. L. Tal'roze along with his collaborators has developed a liquid chromatography tandem-mass spectrometer in the 1970s. They connected liquid chromatography columns with mass spectrometer ion sources via capillaries (de Koster and Schoenmakers 2020; Korfmacher 2007). Fraction collection of eluted analytes, evaporation of solvents and transfer of analytes to a mass spectrometer for analysis are the basic steps involved in a liquid chromatography-mass spectrometer (Korfmacher 2007). In detail, liquid chromatography allows distribution of a liquid mixture in two immiscible phases such as stationary and mobile phase. A stationary phase of non-polar nature and a mobile phase of polar nature are commonly used for the physical separation of components. Liquid solvent of the mobile phase is delivered into the stationary phase via a packed column under high pressure.

Eluted fractions from liquid chromatography undergo ionization in an ion source of a mass spectrometer. Electric or magnetic fields in the mass analyzer of mass spectrometer sort ions based on masses whereas the detector measures and amplifies ion current for calculation of each mass-resolved ion abundance. The result of such an organized process is reflected in form of a mass spectrum to determine m/z ratio of each ion.

At present, various LC-MS/MS are commonly available which are mainly composed of one ion source, for example, mostly nanoESI or ESI and two or more analyzers in it. Thus, a combination of mass spectrometers varies on basis of sensitivity, resolution, specificity, accuracy and used for various applications. These combinations allow to acquire MS and MS/MS spectra which provides better confidence in protein identification. Some of the combinations are discussed below.

Triple-Quadrupole Mass Spectrometer

In the late 1970s, Enke and Yost of Michigan State University have developed the first commercial triple-quadrupole mass spectrometer (TQMS or QqQ) (Yost and Enke 1978). Arrangement of three quadrupoles such as quadruple 1 (Q1), quadrupole 2 (Q2) and quadrupole 3 (Q3) has led to such nomenclature. While Q1 and Q3 are two mass filters with four parallel rods, Q2 is known as a collision cell. In addition, both Q1 and Q3 are supplied with direct current and radiofrequency potential whereas Q2 is controlled by only radiofrequency potential. Both direct current and radiofrequency potential voltages act together in the selectivity of ions based on the m/z ratio (Parker et al. 2010).

After ionization in an ion source, selection of primary ions occur in the first quadrupole where ions with an m/z ratio other than selected ones will not be allowed to infiltrate. In second quadrupole, fragmentation of sample and formation of further daughter ions occurs by collision between a sample and inert gas. The daughter ions enter into quadrupole 3 and are selected on the basis of m/z (Johnson et al. 1990). Ionization, primary mass selection, collision-induced dissociation, mass analysis of fragment ions and detection occur in separate segments of a triple-quadrupole mass spectrometer (Johnson et al. 1990). Therefore, a triple-quadrupole mass spectrometer is highly efficient and has capabilities of a tandem mass spectrometer (Morrison 1991).

Quadrupole-Ion Trap Mass Spectrometer

Another combination of a mass spectrometer known as Quadrupole ion trap mass spectrometer (Q-Trap MS) is composed of both quadrupole and ion trap mass analyzers with electrospray ionization as an ion source (Sandra et al. 2004).

Quadrupole-Orbitrap Mass Spectrometer

Quadrupole orbitrap mass spectrometer was invented by Makarov and consists of two mass analyzers (Makarov et al. 2006a, b). The first analyzer is a quadrupole whereas the second analyzer is a high-resolution orbitrap. Orbitrap consists of two electrodes such as an outer electrode and an inner electrode to trap ions (Hu et al. 2005; Makarov 2000). A balance between electrostatic attraction of ions as well as their inertia leads to ion trapping and ion movement in elliptical trajectories. Detected current from trapped ions are transformed to a mass spectrum based on a fourier transform of frequency signal.

Advancements associated with orbitrap include 150,000 mass resolution, 2–5 ppm mass accuracy, m/z range up to 6000 da, and large space charge capacity (Hu et al. 2005).

Quadrupole Time-of-Flight Mass Spectrometer

Quadrupole time-of-flight (Q-TOF-MS) is a combination of quadruple technologies with a TOF mass analyzer and ESI as an ion source and it was introduced in 2001. Q1 operates as a mass filter for specific ion selection according to the m/z ratio and Q2 carries out fragmentation of ions by collision-induced dissociation (CID) process. After fragmentation, ions are reaccelerated into a TOF analyzer and get pulsed by an electric field and subsequently detected by a detector (Chernushevich et al. 2001).

Matrix-Assisted Laser Desorption Ionization-Time-of-Flight/Time-of-Flight Mass Spectrometer

Gradually, combined application of MALDI-MS and TOF technology lead to a more sensitive technique known as MALDI-TOF/MS. In 1993, a dual analyzer time of flight (TOF/TOF) mass spectrometer along with MALDI was reported. A new reflector, curved-field reflectron (CFR) was developed for improvisation of energy focusing ions generated from time-of-flight (TOF) after initial acceleration. Modifications in tandem TOF/TOF mass spectrometer allows utilization of full kinetic energy from accelerated ions that comes in form of collision energy (Cornish and Cotter 1993b). Tandem TOF/TOF mass spectrometer also effectively obtains post-source decay (PSD) product ion mass spectra (Cordero et al. 1995; Cornish and Cotter 1994).

Modifications in tandem TOF/TOF mass spectrometer allows utilization of full kinetic energy from accelerated ions that comes in form of collision energy (Cornish and Cotter 1993b). Tandem TOF/TOF mass spectrometer also effectively obtains post-source decay (PSD) product ion mass spectra (Cordero et al. 1995; Cornish and Cotter 1994).

3.3 Proteomics Approaches

Quantitative proteomics approaches have been classified into gel-based and non-gel-based approaches which are further sub-classified as label-free and labeling approaches as shown in Fig. 3.3. Earlier, separation of proteins on 1D-PAGE was commonly used and a combination of 1D-PAGE with mass spectrometry became a gel-based approach. Eventually, to overcome the limitations of this approach, non-gel-based approaches based on advancements of mass spectrometry have been invented. Label-free approaches carry out quantification on the basis of spectral counting and ion intensity (Filiou et al. 2012). Labeling approaches rely upon metabolic, enzymatic or chemical labeling strategies (Ross et al. 2004; Thompson et al. 2003). While label-free proteomics approach provides the highest proteome

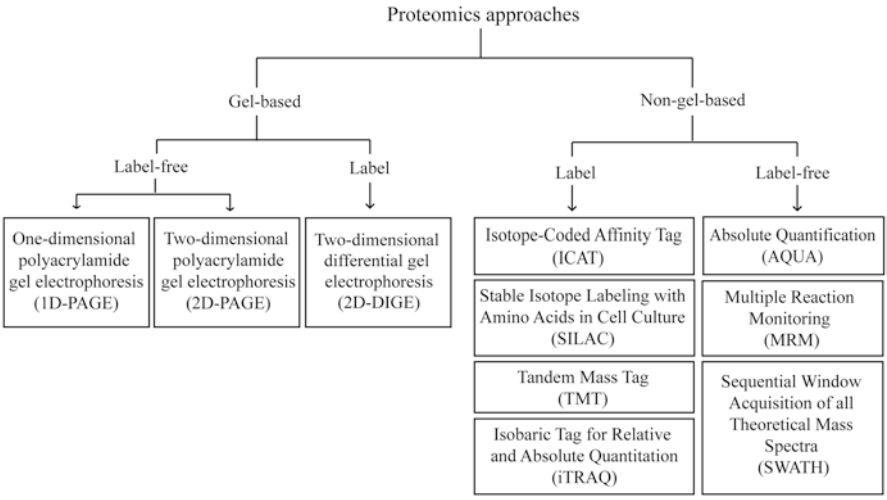

Fig. 3.3 Flow chart showing the classification of quantitative proteomics approaches. These approaches are broadly classified into gel-based and non-gel-based approaches. One-dimensional polyacrylamide gel electrophoresis (1D-PAGE), and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) are the gel-based label-free proteomics approaches whereas two-dimensional differential gel electrophoresis (2D-DIGE) is known as gel-based label proteomics approach. Similarly, Isotope-coded affinity tag (ICAT), Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC), Tandem Mass Tag (TMT), and Isobaric tag for Relative and Absolute Quantitation (iTRAQ) are non-gel-based label proteomics approaches whereas Absolute Quantification (AQUA), Multiple Reaction Monitoring (MRM) and Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) are non-gel-based label-free proteomics approaches

coverage, labeling approach exhibits higher accuracy of protein or peptide quantification (Megger et al. 2014).

3.3.1 Gel-Based Label-Free Proteomics Approaches

3.3.1.1 One-Dimensional Polyacrylamide Gel Electrophoresis

In 1970, one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) was developed by Ulrich K. Laemmli to separate proteins based on their molecular weight. The use of sodium dodecyl sulfate (SDS) masks intrinsic charge of proteins, as a result, intrinsic charge of proteins become negligible. Thus, upon application of electric field, proteins migrate solely according to molecular weight (Laemmli 1970). The standardized workflow of 1D-PAGE is shown in Fig. 3.4a.

First, protein separation on SDS-PAGE is carried out, then gels are stained with either silver nitrate stain or coomassie brilliant blue stain. Post staining, gels are destained for visualization of separated proteins which are observed in form of

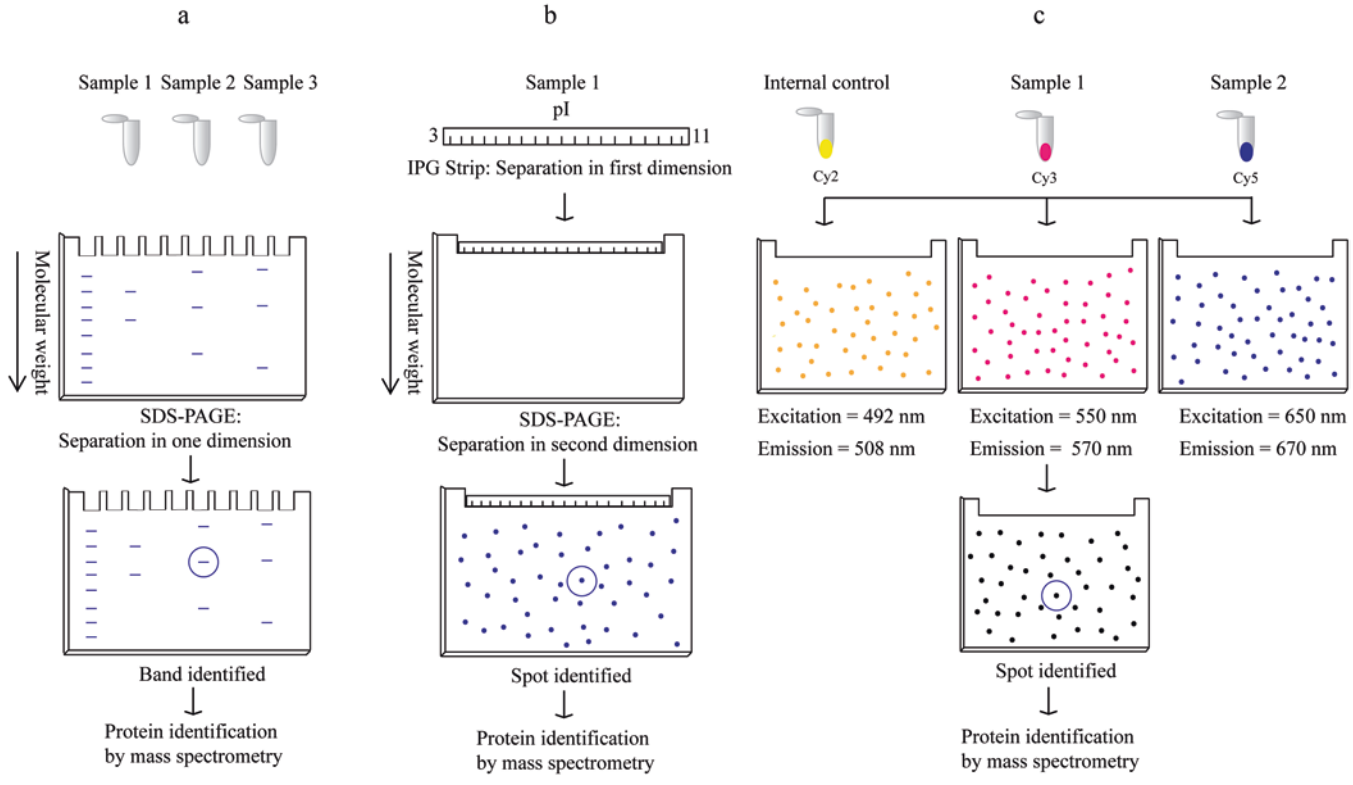

Fig. 3.4 Standardized workflows for gel-based label-free proteomics approaches (**a**) one-dimensional polyacrylamide gel electrophoresis (1D-PAGE), (**b**) two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and gel-based label proteomics approach (**c**) two-dimensional differential gel electrophoresis (2D-DIGE) are shown

bands. The appropriate molecular weight of each protein is determined concerning a standard molecular marker (Laemmli 1970).

Even though, 1D-PAGE is an analytical method to separate proteins based on molecular mass, protein isoforms with similar molecular weight are difficult to resolve by this approach

3.3.1.2 Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was invented by O'Farrell and Klose independently in 1975. More than one thousand different protein spots from *Escherichia coli* were successful through two-dimensional polyacrylamide gel electrophoresis (O'Farrell 1975). This technique separates proteins from complex biological mixtures based on two dimensions, i.e. isoelectric point and molecular weight (Görg et al. 1985; O'Farrell 1975). Immobilized pH gradient (IPG) strips are used in the first dimension to separate proteins based on isoelectric point whereas further separation occurs as per their molecular weight on SDS-PAGE similar to 1D-PAGE. Proteins can distinctly be observed on gels as several well-resolved spots after staining either with coomassie brilliant blue or silver nitrate stain (Westermeier 2016). The standardized workflow of 2D-PAGE is shown in Fig. 3.4b.

2D-PAGE is a proteomics technique suitable for the quantitative assessment of electrophoretically separated proteins. Characterization and resolution of protein isoforms, as well as proteins with post-translational modifications, can be carried out with 2D-PAGE technique (Lee et al. 2020). Post-translational modifications confer change in mass, as well as the charge of protein and such changes in mass and charge, make protein resolve separately by 2D-PAGE technique (Graves and Haystead 2002). Furthermore, comparative analysis of variable protein expression for different biological conditions can be achieved by 2D-PAGE (Pomastowski and Buszewski 2014).

Despite several advantages, 2D-PAGE is a time-consuming and laborious process with an analysis of a single sample per gel. (Celis and Gromov 1999; Graves and Haystead 2002). Hydrophobic and alkaline proteins remain underrepresented by 2D-PAGE (Nilsson et al. 2000). Moreover, proteins with extreme acidity (pH 3) and extreme basicity (pH 10) are unable to represent properly through 2D-PAGE (Görg et al. 2000). The presence of low vital range and gel-to-gel variations are some of the limitations associated with 2D-PAGE (Cho 2007). Inability of low-copy protein detection is another limitation associated with 2D-PAGE as observed with yeast protein analysis (Gygi et al. 1999b).

3.3.2 Gel-Based Label Proteomics Approaches

3.3.2.1 Two-Dimensional Differential Gel Electrophoresis

Few limitations such as lack of reproducibility and quantitation of traditional 2D-PAGE gave rise to another gel-based labeling technique known as two-dimensional differential gel electrophoresis (2D-DIGE). 2D-DIGE was introduced by Unlu and group in 1997 as a modification to 2D-PAGE (Unlü et al. 1997). 2D-DIGE is an improvised quantitative differential proteomics technique of protein separation through pre-labeling them with fluorescent dyes namely Cy2, Cy3 and Cy5 of different spectral properties. After mixing different samples with fluorescent dyes in equal ratio, the mixture is run as one sample on a gel to allow co-migration of similar kind of proteins with different fluorescent dyes and to identify them as one spot position. The gel images specific to different Cy dyes are captured using appropriate wavelengths and visible protein spot specific to the sample are analyzed with an appropriate computer program with different wavelengths where an abundance of protein is calculated after imaging of the gel (Magdeldin et al. 2014; Westermeier 2016). Cy dyes, named after cyanine dyes with an N-hydroxysuccinimidyl ester group that binds to epsilon (ε)-amino groups of lysine residues in proteins. Dyes are added at low concentrations so that one dye molecule can be added per protein. In 2D-DIGE, usually, Cy2-labeled pooled sample is used for normalization pool, formed by mixing all the samples of an experiment in an equal quantity. Cy2-labeled pool is used for normalization of signals and spot matching by providing a consistent spot map in all gels of an experiment (Diez et al. 2010). The standardized workflow of 2D-DIGE is shown in Fig. 3.4c.

Advancement of 2D-DIGE lies in separating fluorescently labeled protein samples on same gel, therefore, minimizes gel-to-gel variations between experimental conditions (Alban et al. 2003). 2D-DIGE is a comparative proteomics technique that enhances both reproducibility and reliability of differential protein expression between samples based on distinct excitation and emission spectra of fluorescent dyes (Meleady 2018). DIGE experiments are favorable for accommodating sufficient biological replicate samples avoiding interpersonal variation among biological samples (Diez et al. 2010). Increased throughput, ease of utilization, reproducibility as well as accurate quantitation in differential protein expression levels has resulted in 2D-DIGE as a more advanced technique compared to 2-DE (Meleady 2018). Thus, 2D-DIGE is helpful in measurement of proteins with less than 5% expression levels with an improved confidence level of results due to obsolete technical replicates (Westermeier 2016). Moreover, the number of gels that need to be compared are reduced in 2D-DIGE with normalization signal abundances among them to correct any differences in signal intensity. Therefore, 2D-DIGE offers a consistent expression profile among gels, focuses on underlying differences, thereby is the most reliable quantitation to describe differences among several biological states (Diez et al. 2010).

Even 2D-DIGE has some limitations. The separation of hydrophobic proteins remains a challenge for 2D-DIGE. In addition, proteins with accessible lysine or cysteine residues are required for minimal labeling whereas cysteine residues are essential for saturation labeling (Drabik et al. 2016).

3.3.3 Non-Gel-Based Label Proteomics Approaches

There are few techniques which are labeling approaches combined with non-gel-based approaches. Some of the most commonly used techniques are described below.

3.3.3.1 Isotope-Coded Affinity Tag

First application of chemical-labeling in quantitative proteomics approach was introduced by Dr. Rudolf Aebersold in 1999 and named as isotope-coded affinity tag (ICAT) technology (Bottari et al. 2004). Isotope-coded affinity tag involves cysteine-specific tags to bind intact proteins of a biological sample. Labeled proteins undergo proteolytic cleavage to give rise to ICAT-labeled peptides (Wiese et al. 2007).

Isotope-coded affinity tag has been utilized to quantify oxidative post-translational modifications of cysteine thiols in most commonly mutated human oncogene p21ras (Sethuraman et al. 2007). Differential host protein expression following hematopoietic necrosis virus infection has been studied with isotope-coded affinity tag technology (Booy et al. 2005). Utilization of phosphoprotein isotope-coded affinity tags (PhIAT) are advancements in isotope-coded affinity tag technology for quantitation of phosphopeptides. PhIAT labeling technique is helpful for differential quantitative measurements of O-phosphorylation state of proteins (Goshe et al. 2001). The standardized workflow of ICAT is shown in Fig. 3.5a.

One of the most convenient advantages of isotope-coded affinity tag technology is compatibility of labeling reaction with common reagents of 2D-PAGE (Cho 2007). Relative quantification in isotope-coded affinity tag labeling technology is considered to be highly accurate due to stable isotope dilution techniques (Gygi et al. 1999a).

However, few limitations are associated with chemical labeling technique. Isotope-coded affinity tag technology was unable to fulfill broad applications due to limited robustness. Only relative quantitation can be revealed via ICAT. Moreover, isotope-coded affinity tag technology is only applicable for cysteine-containing proteins. As cysteine content of proteins usually remains low, isotope-coded affinity tag technology is not reliable for most of the proteins or in other words cysteine-free proteins (Cho 2007; Wiese et al. 2007).

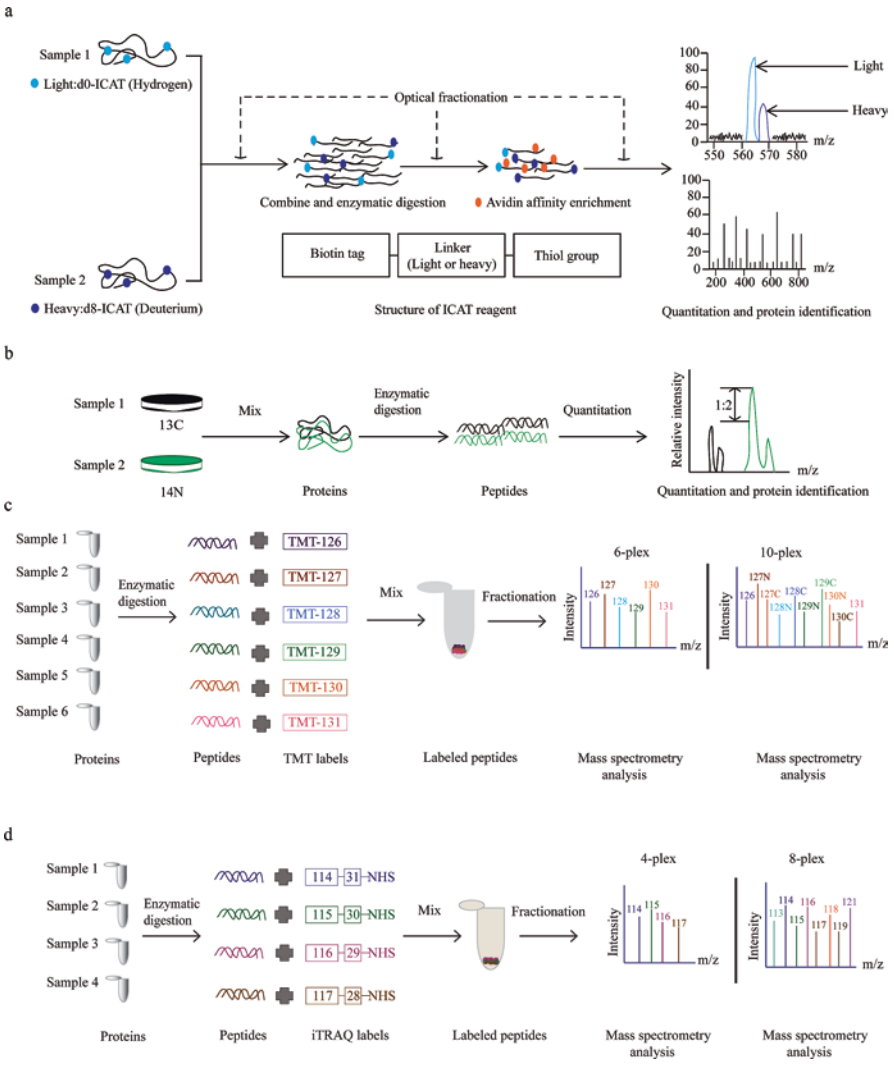

Fig. 3.5 Standardized workflows for non-gel-based label proteomics approaches (**a**) Isotope-coded affinity tag (ICAT), (**b**) Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC), (**c**) Tandem Mass Tag (TMT), and (**d**) Isobaric tag for Relative and Absolute Quantitation (iTRAQ) are shown

3.3.3.2 Stable Isotope Labeling with Amino Acids in Cell Cultures

In a biological sample, 8% of proteins are non-cysteine proteins, therefore, isotope-coded affinity tag technique is unable to detect such proteins. To overcome limitations of ICAT, stable isotope labeling with amino acids in cell culture (SILAC) developed in 2002 by S.E. Ong (Ong et al. 2002). In SILAC, heavy metabolic labels

such as ^{13}C and ^{15}N-labeled amino acids are added into cell culture. As a result, such labels will be incorporated into all synthesized proteins. Before protein extraction, cells with ^{13}C labels and ^{15}N-labeled amino acids are mixed to minimize the technical variation of sample processing. After protein extraction and enzymatic digestion, mass spectrometry analysis reflects ratio of peak intensities to identify protein abundance in cells labeled with either ^{13}C or ^{15}N-labeled amino acids (Mann 2006). The standardized workflow of SILAC is shown in Fig. 3.5b.

SILAC is an advanced approach to identify and quantify of relative expressional changes in protein samples. High compatible nature in all cell culture conditions and complete incorporation of heavy amino acid labels do not affect morphology and growth rates of cells. Furthermore, neither chemical labeling nor affinity purification steps are needed to study the proteome of cell culture (Ong et al. 2002). Despite several advantages, SILAC approach can be used only in cell culture conditions.

3.3.3.3 Tandem Mass Tag

Tandem mass tags (TMTs) are chemical labeling reagents used to identify and quantify peptides and proteins. TMT labeling was introduced by Thomson in 2003 with two tandem mass tags and called as TMT 2-plex (Thompson et al. 2003). Tandem mass tags are amino-reactive reagents with a reporter as well as a balancer group. The balancer group normalizes overall mass and reporter group is useful for peptide quantification (Bąchor et al. 2019).

Based on number and combinations of heavy isotopes, isobaric tandem mass tags can be of several types such as TMT 2-plex, TMT 6-plex and TMT 10-plex (Bąchor et al. 2019). TMT 2-plex consists of two isobaric tags to detect reporter ions at m/z 126, 127. Similarly, TMT 6-plex identifies reporter ions at m/z 126, 127, 128, 129, 130 and 131. Expansion of TMT 6-plex gave rise to TMT 10-plex by adding four more isotopic tag variants with TMT 6-plex isobaric tags with 6.32 mdalton mass difference between ^{13}C and ^{15}N isotopes (Bąchor et al. 2019; Rauniyar and Yates 2014). The standardized workflow of TMT is shown in Fig. 3.5c.

Modifications in tandem mass tag protein labeling approach have been also successful in the direct quantification of intact proteins. Despite the charge state of isobaric TMT labeled proteins, detection of abundance ratio of protein pairs is not hampered (Hung and Tholey 2012). Identification of unique peptides is not a prerequisite for TMT-labeled proteins (Duncan et al. 2010). Additionally, confident identification of small proteins with molecular size less than 35 kilodalton as well as de novo sequencing of unidentified proteins is possible through tandem mass tag protein labeling technique (Hung and Tholey 2012).

A major limitation of tandem mass tag labeling is isolation and fragmentation of labeled peptide precursors together may give chimeric MS/MS spectra. Such chimeric spectra sometimes hamper the expression level of each peptide (Sturm et al. 2014).

3.3.3.4 Isobaric Tag for Relative and Absolute Quantitation

In contrast to isotope-coded affinity tag and isotope-coded protein labeling method, a major alternative for multiplexed quantitative peptide labeling technique was use of isobaric tag for relative and absolute quantitation (iTRAQ). In 2004, iTRAQ was introduced and used to bind stable isotopes into proteolytic peptides (Ross et al. 2004). iTRAQ involves isobaric tags to label peptides, each isobaric tag is an amino-reactive reagent with a reporter group and a balancer group. The reporter group is based on N-methylpiperazine whereas the balancer group is a carbonyl group. Although individual reporter group and balancer group hold variability in mass, their combined mass remains same, therefore named as isobaric tags (Gevaert et al. 2008). Isobaric tag labeling approach exploits N-hydroxy succinimide (NHS) ester derivatives to modify primary amino groups of peptides. The binding of isobaric tags to primary amino groups of peptides forms amide bonds (Ross et al. 2004). Due to presence of isobaric tags, labeled peptides are visualized as one peak in MS scans. However, during MS/MS analysis, bond between the reporter group and balancer group breaks down releasing the balancer group as a neutral fragment. As a result, reporter ions are used for peptide quantification (Gevaert et al. 2008).

iTRAQ has ability to compare multiple samples in a single experiment called multiplexing. iTRAQ with four isobaric tags with mass range of 114, 115, 116 and 117 dalton is known as 4-plex (Ross et al. 2004). and eight isobaric tags with reporter ion having masses 113, 114, 115, 116, 117, 118, 119 and 121 dalton is known as 8-plex (Pierce et al. 2008). Appearance of single peak in MS scan due to use of isobaric tags reduces probability of peak overlapping (Wiese et al. 2007). Apart from protein quantification, discovery of protease substrates is another significant advancement of iTRAQ (Dean and Overall 2007; Enoksson et al. 2007). The standardized workflow of iTRAQ is shown in Fig. 3.5d.

Isobaric-labeling-based proteomics approach has many advantages. Sample multiplexing is one of the major benefits of isobaric-labeling techniques as it infers high-throughput quantification. Combination and analysis of many samples in one experiment eliminates time and run-to-run variation (Bąchor et al. 2019). Distribution in profiling of peptide length as well as content of amino acid in isobaric tagged peptides do not allow interference with peptide fragmentation (Aggarwal et al. 2006). In fact, an increase in efficiency of MS/MS fragmentation along with native peptide signal intensities have been reported in isobaric tagged peptides of complex human samples (Hardt et al. 2005).

Despite several advantages, a major drawback of iTRAQ is enzymatic digestion of proteins before labeling. Such enzymatic digestion increases sample complexity with one or two order of magnitude (Wiese et al. 2007). iTRAQ has the potential to identify high abundant proteins that reflects limited effectiveness of isobaric tag labeling technique in peptide separation. Thus, probability for identification of low abundant proteins gradually decreases with increase in complexity of samples (DeSouza et al. 2005; Wiese et al. 2007). Therefore, iTRAQ is thought to be useful for samples with low to moderate complexity. Furthermore, peptides only subjected

for MS/MS analysis will be provided with quantitative information (Wiese et al. 2007).

However, isobaric peptide labeling techniques have some drawbacks. Isobaric tags are highly expensive and sometimes show variability in labeling efficiencies. Due to involvement of several samples in one experiment, sometimes inconvenient handling becomes a problem (Bąchor et al. 2019). Therefore, label-free quantitative/targeted proteomics approaches have emerged over time.

3.3.4 Non-Gel-Based Label-Free Proteomics Approaches

3.3.4.1 Absolute Quantification

In 2001, Steve Gygi has discovered a targeted proteomics approach known as absolute quantification (AQUA) where stable-isotope labeled peptides are used as internal standards (Stemmann et al. 2001). AQUA approach has two stages such as method development and application of the method into wide range of biological samples. During method development stage, peptides from signature protein are selected and synthesized by incorporation through stable isotopes like ^{13}C, ^{2}H or ^{15}N which serve as an ideal internal standard. The standardized workflow of AQUA is shown in Fig. 3.6a. Synthesis of peptides can also possible by covalent modifications such as phosphorylation, methylation and acetylation. In the application stage, such internal standard peptides are used for absolute quantification of proteins and post-translationally modified proteins by selected reaction monitoring analysis by a tandem mass spectrometer (Kettenbach et al. 2011). Inter-strain genome variation is a major drawback associated with AQUA approach.

3.3.4.2 Multiple Reaction Monitoring

Multiple Reaction Monitoring (MRM) is a targeted proteomics approach used for peptide quantitation and validation (Unwin et al. (2005). Sample is introduced into a triple quadrupole mass analyzer, after which, ionization and mass selection occurs. Design of transitions for target protein with selection of peptides (otherwise known as precursor ions) and respective product ions is critical for an MRM based experiment (Lange et al. 2008). Based on pre-designed transition lists, peptide selection (precursor ion selection) occurs in quadrupole 1 of mass ions. Quadrupole 3 carries out mass selection where a specific fragment ion is selected for analysis with predefined m/z (McKay et al. 2007). The standardized workflow of MRM is shown in Fig. 3.6b.

Use of two selection steps has ensured high specificity of MRM approach. Therefore, specific target of a protein or peptide is possible through MRM for both relative and absolute quantitation purposes (Kondrat et al. 1978). The advantage of MRM associates with non-requirement of antibody and thus saves time required for

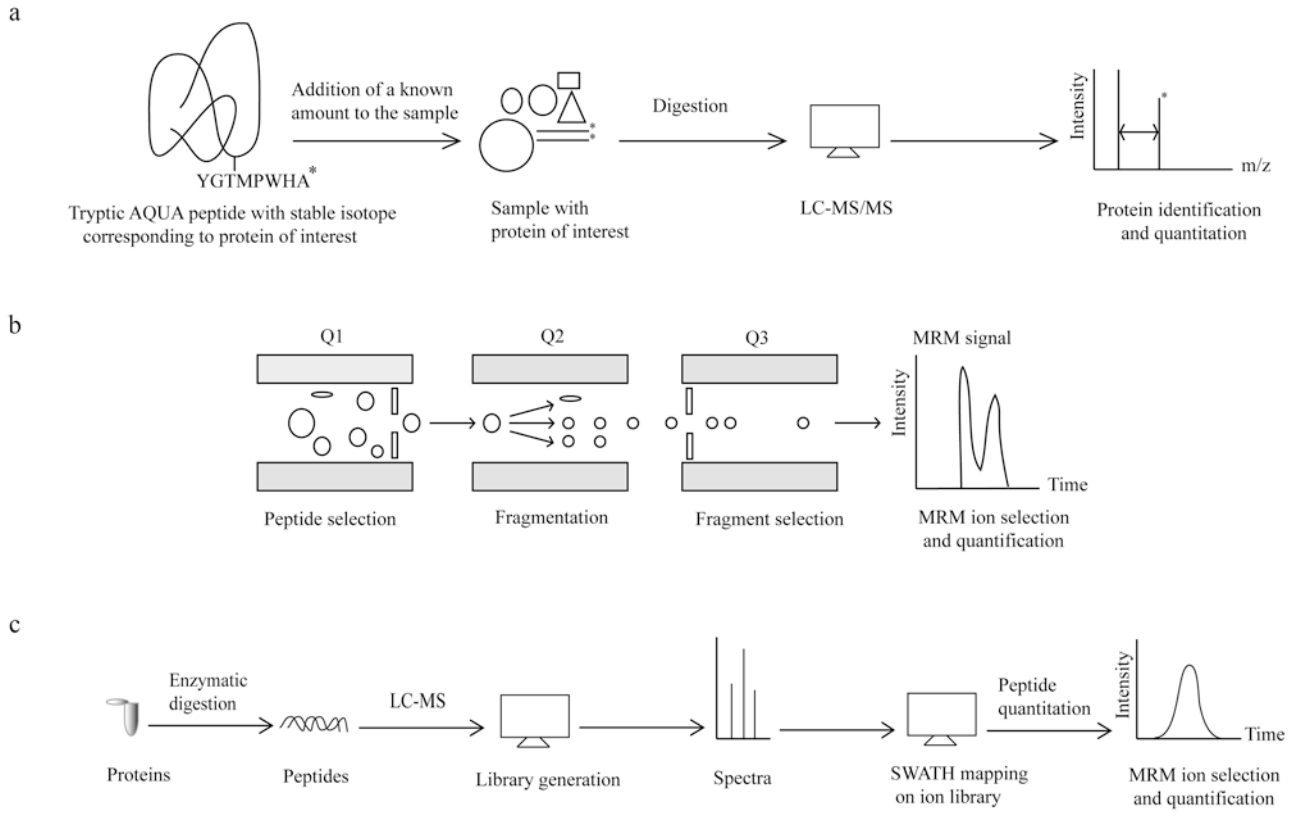

Fig. 3.6 Standardized workflows for non-gel-based label-free proteomics approaches (**a**) Absolute Quantification (AQUA), (**b**) Multiple Reaction Monitoring (MRM) and (**c**) Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) are shown

antibody development. Furthermore, simultaneous quantification of up to forty-five proteins is possible through MRM approach (Kuzyk et al. 2009).

3.3.4.3 Sequential Window Acquisition of All Theoretical Mass Spectra

Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) is a more recent proteomics approach developed in 2011 by Genna L Andrews (Andrews et al. 2011). SWATH is based upon data independent acquisition for high throughput protein quantification (Huang et al. 2015). Generation of spectral library is the first step of SWATH for identification of proteins acquired in SWATH-MS runs. However, if peptide information is available in database such as Peptide Atlas, then generation of spectral library is not necessary. Data acquisition of each sample under analysis is the second step of SWATH approach where a quadrupole time-of-flight mass spectrometer is required to fulfill typical conditions such as 400–1200 da m/z in thirty-two steps at 25 m/z widths. Matching of individual SWATH-MS run against spectral library is the third step for peptide identification in a data-independent manner. In the final step, specific peptide ions are extracted to enable area-under-the-curve quantitation between samples (Gillet et al. 2012). The standardized workflow of SWATH is shown in Fig. 3.6c.

Unmatched selectivity, sensitivity, reproducibility, coverage and dynamic quantitation range are the advantages of SWATH proteomics approach. However, the requirement of a specific high-resolution mass spectrometer with data independent analysis ability is a disadvantage at present. Different proteomics approaches with their advantages and limitations are summarized in Table 3.2.

3.4 Proteomics of Animal Viruses

Viruses are infectious agents, capable to carry out by successful adaptation and modulation of the host environment and cause diseases. Chronologically, while viruses were first to be known with complete genome sequences, corresponding proteome of viruses has been elucidated recently (Viswanathan and Früh 2007). In comparison to genome of a cell, proteome is more functionally relevant and usually changes due to interaction with genome, different stages of lifecycle or variable environmental conditions. Viruses are obligate parasites dependent upon host's machinery for their propagation. At the same time, virus infection alters several cellular pathways that can be reflected through proteome of a cell (Coombs et al. 2010).

Viral proteins play a major role during infection and share contribution in host-cell interactions. Investigations on involvement of specific viral proteins during pathogenesis are a pre-requisite for functional studies. Therefore, several proteomics studies have emerged to investigate protein composition of viruses, interaction among viral proteins or host-viral proteins along with effect of viral proteins on cellular proteome (Maxwell and Frappier 2007).

Table 3.2 Different proteomics approaches with their advantages and limitations

Serial no.	Proteomics approach	Advantages	Limitations
1	1D-PAGE	Electrophoretically separates proteins on basis of molecular weight	Difficult to separate proteins of same molecular weight and isoforms
			Has poor reproducibility
2	2D-PAGE	Electrophoretically separates proteins on basis of isoelectric point and molecular weight	A time consuming and labor-intensive process
		Comparative analysis between different samples	Analysis of only one sample per gel
		Characterization and resolution of protein isoforms as well as proteins with post-translational modifications	Difficult to completely resolve proteins from complex sample
			Underrepresentation of hydrophobic proteins
			Inability to resolve proteins of extreme acidity and extreme basicity
			Low dynamic range and gel-to-gel variability
3	2D-DIGE	Separates fluorescently labeled protein samples on the same gel	Difficult to separate hydrophobic proteins
		Minimizes gel-to-gel variations between multiple experiments	Minimal labeling is possible only for proteins with accessible lysine or cysteine residues
		Enhances both reproducibility and reliability of differential protein expression	Proteins with only cysteine residues are essential for saturation labeling
		Favourable for accommodating biological replicate samples	
		Most sensitive gel based approach. Hence, protein quantitation is reliable.	
4	ICAT	Labeling reaction is compatible with common reagents of 2D-PAGE	Unable to fulfil broad applications due to limited robustness
		Relative quantification is highly accurate	Only applicable for cysteine-containing proteins
5	SILAC	Metabolic incorporation of ^{13}C or ^{15}N-labeled amino acids occurs into proteins	Can be used only in cell culture

(continued)

Table 3.2 (continued)

Serial no.	Proteomics approach	Advantages	Limitations
6	TMT	Direct quantification of intact proteins is possible due to the isobaric labels	Enzymatic digestion of proteins is required before labeling
		Detection of abundance ratio of protein pairs is not hampered	Co-isolation and co-fragmentation of labeled peptide precursors may give chimeric MS/MS spectra
		Multiplexing of sample in a single experiment is possible.	
		Identification of unique peptides is not a pre-requisite for labeled proteins	Chimeric spectra sometimes hamper expression level of each peptide
		Confident identification of small proteins with molecular size less than 35 kDa is possible	Tags are highly expensive and sometimes show variability in labeling efficiencies
		de novo sequencing of unknown proteins is possible	
7	iTRAQ	Compares multiple samples in a single experiment	Enzymatic digestion of proteins is required before labeling
		Reduces probability of peak overlapping	Enzymatic digestion increases sample complexity with one or two order of magnitude
		Discovery of protease substrates is possible	Useful for samples with low to moderate complexity
		Allow better protein/peptide quantitation	Tags are highly expensive and sometimes show variability in labeling efficiencies
8	AQUA	Stable isotope-labeled peptides are used as internal standards	Inter-strain genome variation occurs
		Causes precise and quantitative measurement of absolute levels of proteins and post-translationally modified proteins	
9	MRM	Peptide quantitation and validation is possible	This is a targeted proteomics approach, hence, not suitable for discovery.
		Absolute quantification without need of antibody	Not suitable for very low abundant proteins.
		A highly specific, sensitive and quantitative approach	
10	SWATH	A high throughput, label-free protein quantification technique	Information about acquired peptide is dependent upon spectral library
		Generated spectral library provides necessary information about acquired peptides	
		Area-under-the-curve quantitation is done between samples	Need specific mass spectrometer and software to analyze the data
		Selectivity, sensitivity, reproducibility, coverage and dynamic quantitation range is unmatched	

In an organism, most cellular pathways are modulated at a proteome level to perform biological function in response to any stimulus. In addition, viral infections alter several functions at protein level, thus, further expand complexity of the proteome. With a challenge of protein function studies during viral infection, modern quantitative mass spectrometry (MS) based proteomics approaches have unambiguously defined alterations in protein biology with respect to localizations, expressions, interactions and modifications (Greco et al. 2014). Hence, current proteomics research has become most promising to provide a better insight into global view of host response upon virus infection (Mehta et al. 2015). In last few years, studies based on proteomics have significantly contributed to investigate alterations in host proteins due to viral infections. These studies have enriched the area of viral proteomics (Munday et al. 2012).

Animal viruses are broadly classified into three major categories; (1) animal viruses, mostly affecting animal species, (2) human viruses, known to affect humans and (3) zoonotic viruses, mostly affecting both animals and humans as shown in Fig. 3.7. This section of the chapter reviews the proteomics studies carried out to understand various aspects of animal viruses. This section also discusses about the identified proteins and their role in viral infection. Some of the commonly observed viruses and their proteomics studies are described here. Summary of proteomics studies carried out for various virus infections are described in Tables 3.3a, 3.3b, 3.3c and list of identified proteins using proteomics approaches in various virus infections along with their role in virus infections are described in Tables 3.4a, 3.4b, and 3.4c.

3.4.1 Animal Viruses

3.4.1.1 Classical Swine Fever Virus

Classical swine fever virus (CSFV) is an enveloped, single positive-strand RNA virus with genus *Pestivirus* of *Flaviviridae* family (Edwards et al. 2000). According to World Organization for Animal Health (OIE), classical swine fever virus causes a contagious disease in pigs known as classical swine fever (Sun et al. 2011). Skin haemorrhages as well as mucosal and internal organ haemorrhages in pigs are major symptoms of classical swine fever (Moennig and Plagemann 1992). Furthermore, following classical swine fever virus infection, a significant decrease in peripheral B cells and T cells occurs due to bystander apoptosis observed in uninfected cells (Summerfield et al. 1998; Susa et al. 1992).

To date, very few proteomics studies are reported with classical swine fever virus. In classical swine fever virus-infected porcine kidney 15 (PK-15) cells, proteomics approach 2D-PAGE with MALDI-TOF/MS has shown alterations in expression of thirty-five protein spots in comparison to uninfected cells. However, twenty-one proteins were successfully detected by mass spectrometry analysis. Out of twenty-one proteins, up-regulation of sixteen proteins and down-regulation of five proteins were observed (Sun et al. 2008). Major alterations have been observed

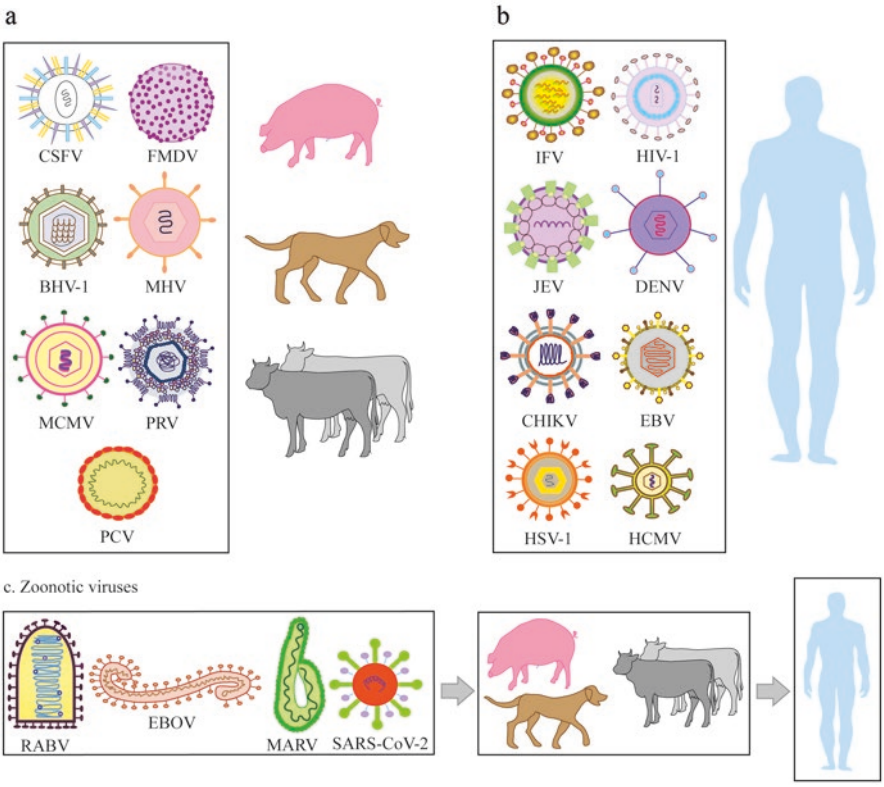

Fig. 3.7 Major animal viruses, classified into (**a**) animal viruses, (**b**) human viruses and (**c**) zoonotic viruses. Animal viruses include Classical swine fever virus (CSFV), Foot-and-mouth disease virus (FMDV), Bovine herpes virus 1 (BHV-1), Murine herpes virus (MHV), Murine cytomegalovirus (MCMV), Pseudorabies virus (PRV), and Porcine circovirus (PCV); Human viruses include Influenza virus (IFV), Human immunodeficiency virus type 1 (HIV-1), Japanese encephalitis virus (JEV), Dengue virus (DENV), Chikungunya virus (CHIKV), Epstein barr virus (EBV), Herpes simplex virus 1 (HSV-1), and Human cytomegalovirus (HCMV) and Zoonotic viruses include Rabies virus (RABV), Ebola virus (EBOV), Marburgvirus (MARV) and Severe acute respiratory syndrome-corona virus-2 (SARS-CoV-2)

in host proteins of cellular functions like energy metabolism, signal transductions, replication, transcription and translation processes (Sun et al. 2008).

Upon classical swine fever virus infection, annexin 2 (annexin A2) protein undergoes up-regulation (Sun et al. 2008). Annexin 2 plays role in viral entry, replication, assembly, budding and release. Infection of human immunodeficiency virus in monocyte-derived macrophages has shown annexin 2 mediated viral entry via interaction with phosphatidylserine (Ma et al. 2004). Furthermore, annexin 2 also serves as an important receptor of respiratory syncytial virus (Malhotra et al. 2003). Therefore, up-regulation of annexin 2 in classical swine fever virus infection may

Table 3.3a Summary of proteomics studies carried out for various animal virus infections

Serial no.	Virus name	Model system used	Proteomics technique used	Major outcome	References
1	Classical swine fever virus	Infected and uninfected PK-15 cells	2D-PAGE with MALDI-TOF/MS	Identification of 21 differentially expressed proteins including 5 down-regulated and 16 up-regulated proteins	Sun et al. (2008)
		PBMCs from infected and uninfected pigs	2D-PAGE with MALDI-TOF/MS	Identification of 34 unique proteins	Sun et al. (2010)
		Serum of infected and uninfected pigs	2D-DIGE with MALDI-TOF/ MS	Identification of 10 proteins with 6 down- regulated and 4 up-regulated proteins	Sun et al. (2011)
2	Foot and mouth disease virus	Infected and uninfected IBRS-2 cells	SILAC with LC-MS/MS	Identification of 127 differentially expressed proteins with 50 down and 77 up-regulated proteins	Ye et al. (2013)
		Infected and uninfected BHK-21 cells	SILAC with LC-MS/MS	Identification of 153 differentially expressed proteins including 96 down and 57 up- regulated proteins	Guo et al. (2015a)
3	Bovine herpesvirus 1	Virulent and attenuated virus infected and uninfected MDBK cells	2D-PAGE with MALDI-TOF/MS	Identification of 8 differentially expressed proteins	Guo et al. (2015b)
		HSV-1 infected HaCaT cells and BHV-1 infected MDBK cells	1D-PAGE with nanoLC-MS/MS	Identification of 883 host proteins and 51 virus proteins. Out of 51 virus proteins, 9 proteins were unique to bovine herpesvirus 1 whereas 42 proteins were common in herpes simplex virus 1 and bovine herpesvirus 1	Russell et al. (2018)
		Infected and uninfected MDBK cells	2D-PAGE with MALDI-TOF/MS	Identification of few novel proteins like Ribonuclease/angiogenin inhibitor 1, Rab guanosine diphosphate dissociation inhibitor alpha 2	Magalhães-Junior et al. (2020)
4	Murine herpesvirus	Purified virions from infected 293T or NIH 3T3 cells	1D-PAGE with microLC-MS/MS	Identification of 14 viral structural proteins	Bortz et al. (2003)
		Purified virions from infected BHK-21 cells	1D-PAGE with nanoLC-MS/MS	Identification of 31 viral structural proteins	Vidick et al. (2013)
				Identification of host protein annexin A2	

(continued)

Table 3.3a (continued)

Serial no.	Virus name	Model system used	Proteomics technique used	Major outcome	References
5	Murine cytomegalovirus	Purified virions from infected MEFs of C57BL/6 mice	1D-PAGE with nanoLC-MS/MS	Identification of 38 viral proteins	Kattenhorn et al. (2004)
6	Pseudorabies virus	Purified virions from infected porcine kidney cells	2D-PAGE with peptide mass fingerprint by MS	Identification of glycosylation isoforms viral glycoprotein B, multiple isoforms of major viral capsid protein U_L19 and isoforms of heat shock protein 70. Identification of annexin A1 and A2 as host proteins	Michael et al. (2006)
		Purified virions from wild type and mutant virus infected porcine kidney cells	SILAC with peptide mass fingerprint by MS	Mutant strain did not affect viral capsid protein production whereas replenished viral tegument protein with cellular actin protein.	Michael et al. (2006)
		Purified virions from infected PK-15 cells	Two complementary proteomics approaches such as 1D-PAGE with orbitrap MS/MS and in-solution digestion with nanoLC-MS/MS	Identification of 35 viral proteins by both approaches. However, additional 12 viral proteins were detected by only nanoLC-MS/MS approach.	Kramer et al. (2011)
		Brain tissue from C57/BL mice infected with purified recombinant virion	LC-MS/MS	Identification of greater than 2000 proteins at tissue level and greater than 1300 proteins at synaptosome level	Zeng et al. (2018)
7	Porcine circovirus	Infected and uninfected PK-15 cells	SILAC with LC-MS/MS	Identification 163 significantly altered proteins including 84 down-regulated and 79 up-regulated proteins	Fan et al. (2012)
		Infected PK-15 cells	LC-MS/MS	Identification of 222 putative viral capsid interacting host proteins	Zhou et al. (2020)
		Caudal left lung lobe from infected and uninfected piglets	iTRAQ with LC-MS/MS	242 differentially expressed cellular proteins with 142 down-regulated and 100 up- regulated proteins	Jiang et al. (2020)

Table 3.3b Summary of proteomics studies carried out for various human virus infections

Serial no.	Virus name	Model system used	Proteomics technique used	Major outcome	References
1	Influenza virus	Infected and uninfected MDCK and A549 cells	2D-DIGE and nanoLC-MS/MS	Identification of 16 differentially expressed host proteins	Vester et al. (2009)
		Infected and uninfected A549 cells	SILAC with 2D-PAGE and LC-MS/MS	Identification of 280 differentially expressed proteins with 153 down-regulated and 127 up-regulated proteins	Coombs et al. (2010)
		Infected and uninfected A549 cells	Proteome-wide comparative pulse-labeling by combination of AHA and SILAC	Identification of 7729 host proteins and 10 viral proteins	Bogdanow et al. (2019)
2	Human immunodeficiency virus	Purified virions from infected MDMs	LC-MS/MS	Identification of 253 unique proteins with 220 novel proteins.	Chertova et al. (2006)
		Infected and uninfected primary CD4 cells	LC-MS/MS	Identification of 168 cellular proteins	Chan et al. (2009)
		Infected and uninfected HEK293T cells	LC-MS/MS	Identification of 46 host proteins	Lin et al. (2014)
3	Japanese encephalitis virus	Infected and uninfected human neuroblastoma SK-N-SH cells	LC-MS/MS	Identification of a 57 kilodalton protein in association with virus	Liang et al. (2011)
		Infected and uninfected stable porcine kidney cells	MALDI-TOF/MS	Identification of a 60 kilodalton protein molecule	Das et al. (2011)
		Infected and uninfected HeLa cells	SILAC with LC-MS/MS	Identification of 158 differentially expressed proteins	Zhang et al. (2013)
		Infected and uninfected neuro2A cells	2D-PAGE with MALDI-TOF/TOF	Identification of 56 differentially expressed proteins	Sengupta et al. (2014)

(continued)

Table 3.3b (continued)

Serial no.	Virus name	Model system used	Proteomics technique used	Major outcome	References
4	Dengue virus	Serum samples from dengue fever and dengue hemorrhagic fever patients	2D-PAGE with MALDI-TOF/MS	Identification of a 50 kilodalton serine protease inhibitor and a viral protein	Thayan et al. (2009)
		Serum samples from dengue fever patients and healthy controls	2D-DIGE with MALDI-TOF/MS	Identification of 48 differentially expressed proteins with 25 down and 23 up-regulated proteins.	Ray et al. (2012)
		Serum samples from dengue fever patients and healthy controls	TMT based nanoLC-MS/MS	Identification of 441 differentially expressed proteins	Han et al. (2019)
5	Chikungunya virus	Infected and uninfected HFLS cells	Ge-LC-MS/MS	Differential expression of 259 proteins at 12 h and 241 proteins at 24 h post-infection.	Sukkaew et al. (2020)
6	Epstein-barr virus	Infected and uninfected human Burkitt lymphoma B cells	TMT based orbitrap MS/MS	Identification of 69 viral encoded proteins	Ersing et al. (2017)
		Infected and uninfected breast epithelial cells MCF10A	LC-MS/MS	Identification of more than 7700 proteins	DeKroon et al. (2018)
7	Herpes simplex virus 1	Infected and uninfected HeLa cells	nanoLC-MS	Identification of 48 viral proteins and 49 distinct host proteins	Loret et al. (2008)
		Infected and uninfected human SK-N-SH cells	BONCAT based mass spectrometry	Identification of 9 cryptic orphan protein coding sequences	Kato et al. (2020)
		Infected and uninfected HaCaT cells	Quantitative temporal viromics-TMT with orbitrap MS/MS	Identification of 7000 human proteins and greater than 90% of canonical viral proteins	Soh et al. (2020)

8	Human cytomegalovirus	Purified virions from infected primary human fetal fibroblasts	1D-PAGE and subsequent protein sequencing	Identification of 6 viral proteins and 1 host protein	Baldick and Shenk (1996)
		Purified virions from infected human dermal fibroblasts	LC-MS/MS and FTIR mass spectrometry	Identification of 12 novel viral open reading frame encoded proteins	Varnum et al. (2004)
		Purified recombinant virions from infected primary human foreskin fibroblasts	MALDI-LTQ-orbitrap cross-linking mass spectrometry	Identification of binding partners for viral proteins	Moorman et al. (2010)
		Infected and uninfected human fetal foreskin fibroblasts	Quantitative temporal viromics with TMT	Identification of 1200 cell surface proteins	Weekes et al. (2014)
		Infected and uninfected primary human fibroblasts	Quantitative temporal viromics with TMT	Sub cellular organization of 4000 host proteins was observed	Jean Beltran et al. (2016)
		Infected and uninfected primary human MRC-5 fibroblasts	MIB-mass spectrometry	Quantitative measurement of perturbations in greater than 240 cellular kinases	Arend et al. (2017)
		Infected and uninfected primary human fetal foreskin fibroblasts	Quantitative temporal viromics with TMT	Targeting greater than 250 human proteins	Nightingale et al. (2018)
		Infected and uninfected MRC5 primary human fibroblasts	PRM	Identification of 60 peroxisomal proteins	Jean Beltran et al. (2018)

Table 3.3c Summary of proteomics studies carried out for various zoonotic virus infections

Serial no.	Virus	Model system used	Used proteomics technique	Number of major altered/identified proteins	References
1	Rabies virus	Brain tissue from infected ICR mice with uninfected controls	2D-PAGE with MALDI-TOF/MS	Differential expression of more than 55 proteins	Dhingra et al. (2007)
		Infected tissue from hippocampus, brainstem and spinal cord of paralytic and furious dogs with uninfected controls	2D-PAGE with qTOF/MS	Differential expression of 32, 49 and 67 proteins in hippocampus, brainstem and spinal cord respectively	Thanomsridetchai et al. (2011)
		Infected and uninfected human brain tissue	2D-PAGE with MALDI-TOF/MS	Identification of 14 differentially expressed proteins with 13 down-regulated and 1 up-regulated proteins	Farahtaj et al. (2013)
		Infected and uninfected human brain tissue	iTRAQ with LC-MS/MS	Identification of 402 proteins	Venugopal et al. (2013)
		Infected and uninfected dog brain tissue	iTRAQ with nanoLC-MS/MS	Identification of 19 differentially expressed proteins with 10 down regulated and 9 up regulated proteins	Behera et al. (2020)
2	Ebola virus	Purified virion	1D-PAGE with LC-MS/MS	Identification of heat shock protein 70 family member	Spurgers et al. (2010)
		Infected and uninfected plasma from non-human primates	LC-MS/MS and a peptide tagging approach	Identification of acute phase proteins, ribosomal proteins	Ward et al. (2019)
3	Marburg virus	Purified virion	1D-PAGE with LC-MS/MS	No significant differential expression of proteins	Spurgers et al. (2010)

4	Severe acute respiratory syndrome corona virus 2	Infected and uninfected caco-2 cells	mePROD	Identification of 6382 proteins	Bojkova et al. (2020)
		PBMCs from infected patients with healthy controls	AP-LC-MS/MS	Identification of human inflammatory and immune response proteins	Li et al. (2020)
		Infected and uninfected human Huh7 cells	TMT prolabeling with LC-MS/MS	Identification of several signaling pathways	Appelberg et al. (2020)
		Gargle samples of infected patients	nano-HPLC coupled with orbitrap-MS/MS	Identification of unique peptides from viral nucleoprotein	Ihling et al. (2020)
		Nasopharyngeal epithelial swab samples from infected patients	LC-MS/MS	Confident identification of viral nucleoprotein	Nikolaev et al. (2020)
		Serum from infected and uninfected patients	Combination of targeted proteomics and metabolomics assays	Identification of 105 differentially expressed proteins	Shen et al. (2020)
		Nasopharyngeal swab from infected samples	nanoLC-MS/MS	Identification of immune system proteins	Akgun et al. (2020)

Table 3.4a List of identified proteins using proteomics approaches in various animal virus infection studies along with their role in viral infections

Serial no.	Virus name	Major protein/s identified	Role of protein/s	References
1	Classical swine fever virus	Up-regulation of annexin A2	Play essential role in viral replication	Sun et al. (2008)
		Down-regulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Facilitates a metabolic switch to reroute carbohydrate flux and to counteract oxidative stress	
		Alterations in cytoskeletal proteins such as cofilin, moesin, vinculin and talin1	May alter cytoskeletal network and induce apoptosis	Sun et al. (2010)
		Down-regulation of peroxiredoxin-1 (Prx-1)	May decrease cellular resistance against oxidative stress and induce apoptosis	
		Down-regulation of apolipoprotein A-I (apo A-I) and thrombin inhibitor isoform 2	Leads to typical symptom such as disruption in hemostatic balance	Sun et al. (2011)
		Down-regulation of haptoglobin	Hampers angiogenesis and vascular repair	
		Down-regulation of vitamin D-binding protein	Affects host immune response and plays role in viral pathogenesis	
		Up-regulation of retinol-binding protein 4 (RBP4)	Leads to onset of kidney injury due to viral infection	
2	Foot-and-mouth disease virus	Down-regulation of cathepsin D	Causes improper functioning of host immune system and blockage of apoptosis	Ye et al. (2013)
		Up-regulation of cystatin	Plays role in host defense mechanism	
		Down-regulation of protein kinase R	Shows antagonistic relationship with virus with respect to antiviral and antitumor response as well as viral replication	Guo et al. (2015a)
		Down-regulation of vacuolar protein-sorting associated protein 28 (VPS28)	May function to suppress viral infection	
		Up-regulation of aspartyl-tRNA synthetase (ARS)	May play role in regulation of translation machinery	

3	Bovine herpes virus 1	Identification of 8 proteins namely pyruvate kinase, heat shock protein 90 AA1 (HSP90AA1), heat shock protein 90 AB1 (HSP90AB1), annexin A, albumin, scinderin , tubulin and vimentin	Emphasize important role in tumor formation and development, cell migration, tumor cell line apoptosis and cell invasion and viral infection	Guo et al. (2015b)
		Depletion of tegument proteins namely U_L36, U_L37, U_L48, U_L21, U_L16	Play role in bridging of capsid and envelope as well as viral replication	Russell et al. (2018)
		Identification of conservation of 51 viral proteins between bovine herpes virus 1 and herpes simplex virus 1	Involve in maintainence of genome homology between two herpesviruses	
		Down-regulation of ribonuclease/angiogen inhibitor 1 (RNH1)	May play role in host defense mechanism	Magalhães-Junior et al. (2020)
		Down-regulation of Rab guanosine diphosphate dissociation inhibitor alpha 2 (GDI2)	Have some role during early stages of viral infection	
4	Murine herpesvirus	Identification of 1 tegument protein, 2 envelope glycoproteins, 5 nucleocapsid protein homologues, and 6 virion associated proteins	May involve in early phase viral infection or viral assembly and egress of virus	Bortz et al. (2003)
		Identification of 31 viral proteins including 13 tegument proteins, 9 envelope proteins, 8 capsid proteins and 1 unclassified structural protein	Provides insight into structural components of virus	Vidick et al. (2013)
5	Murine cytomegalovirus	Identification two abundant proteins such as tegument protein M25 and major capsid protein M86	Deciphers complex composition of virus	Kattenhorn et al. (2004)
6	Pseudorabies virus	Identification of glycosylation isoforms of viral glycoprotein B (gB) and multiple isoforms of viral capsid protein unique long region 19 (U_L19)	Serve as constituents of viral particle	Michael et al. (2006)
		Identification of multiple isoforms of host proteins annexin A1 and A2 and heat shock protein 70 (HSP 70)	Identified as structural components of virus	
		Lack of viral tegument protein unique long region 3 (U_L3), unique long region 47 (U_L47) or unique long region 49 (U_L49)	Suggests tegument replenishment by cellular actin	

(continued)

Table 3.4a (continued)

Serial no.	Virus name	Major protein/s identified	Role of protein/s	References
		Identification of several viral proteins along with four phosphorylated viral proteins namely unique long region 26 (U_L26), unique long region 36 (U_L36) , unique long region 46 (U_L46) and unique long region 48 (U_L48)	Deciphers potential role in activation of viral proteins during early stages of virus infection	Kramer et al. (2011)
		Identification of co-regulations among host proteins of synaptic transmission, cargos transport and cytoskeleton organization	Play role in virus trafficking	Zeng et al. (2018)
		Down-regulation of shank protein family and snap29	Inhibition of host synaptic transmission	
7	Porcine circovirus	Up-regulation of calpain-1 and ryanodine receptor 1	Together play role in viral pathogenesis	Fan et al. (2012)
		Down-regulation of cytoskeletal proteins	Involve in mechanism of viral pathogenesis	
		Identification of interaction of host proteins such as heterogeneous nuclear ribonucleoprotein C, nucleophosmin 1, DEAD-box RNA helicase 21, importin beta 3, eukaryotic translation initiation factor 4A2, snail family transcriptional repressor 2, MX dynamin like GTPase 2, and intermediate chain 1 with viral capsid protein	Provides insight into viral replication and pathogenesis	Zhou et al. (2020)
		Up-regulation of ATP synthase	A pre-requisite for viral self-proliferation	Jiang et al. (2020)
		Up-regulation of interferon-induced proteins	Activation of interferon signaling pathway to contribute resistance against virus infection	
		Up-regulation of iC3b, collectins, p40phox, major histocompatibility class-I and major histocompatibility class-II	Activation of phagosome pathway for phagocytosis of pathogens	

Table 3.4b List of identified proteins using proteomics approaches in various human virus infection studies along with their role in viral infections

Serial no.	Virus name	Major protein/s identified	Role of protein/s	References
1	Influenza virus	Up-regulation of keratin 10	May facilitate progeny virus transport	Vester et al. (2009)
		Up-regulation of Mx proteins	Play role in signal transduction, cellular stress response and apoptosis	
		Down-regulation of beta catenin	Hamper interferon induction required for antiviral response	Coombs et al. (2010)
		Down-regulation of beta catenin binding protein WD40	Affect several signal transduction pathways and molecular binding processes	
		Down-regulation of transcription factor IIA	Important for interferon and interferon inducible gene transcription	
		Down-regulation of several components of major histocompatibility complex I	May lead to immune cell-mediated attack	
		Induction of viral proteins during 8–12 h pulse interval	Allows both permissive and non-permissive infection	Bogdanow et al. (2019)
		Variability in gradual reduction of host proteins upon human and bird adapted virus strain infection	Post-translational modifications are suspected reason behind such host response in two different viral strain	
2	Human immunodeficiency virus	High dominance of cytoskeleton, adhesion signalling, intracellular trafficking, metabolic, ubiquitin/ proteasomal and immune system proteins	Can serve as potential targets against viral infection	Chertova et al. (2006)
		Up-regulation of G-rich sequence factor 1 (GRSF1)	Maintains stability of viral transcript	Chan et al. (2009)
		Up-regulation of integrin and transgelin	May facilitate virus replication cycle.	
		Identification of DEAD-box RNA helicase1 (DDX1)	Alters subcellular localization of viral Rev protein. Plays role in virus replication.	Lin et al. (2014)
		Identification of B23 and chromosomal maintenance 1 protein (CRM1)	Play role in functional regulation of viral Rev protein	

(continued)

Table 3.4b (continued)

Serial no.	Virus name	Major protein/s identified	Role of protein/s	References
3	Japanese encephalitis virus	Identification of intermediate filament protein vimentin	Play role in virulence of virus	Liang et al. (2011)
		Identification of vimentin	Acts as receptor for virus	Das et al. (2011)
		Up-regulation of interferon-stimulated gene 15 (ISG15), GTPase, Mx1, protein kinase R, 2'-5'-oligoadenylate synthetase	Play role in host immune response	Zhang et al. (2013)
		Up-regulation of interferon-induced transmembrane protein 3 (IFITM3), sterile alpha motif domain-containing protein (SAMD9), vesicle-associated membrane protein 8 (VAMP8)	Shows host antiviral effect against virus infection	
		Up-regulation of heterogenous nuclear ribonucleoprotein H1 (HNRNPH1)	Involve in virus induced invasion of host machinery for viral replication	Sengupta et al. (2014)
		Observation in cytoskeletal perturbations	Act as viral protein reservoir during virus multiplication	
		Up-regulation of host stress response protein ERP29	Enhances host stress response during infection	
		Down-regulation of protein disulphide isomerise family protein ERP57	Affects plasma membrane and nuclear signalling events	
4	Dengue virus	Up-regulation of viral non-structural protein 1 (NS1)	Mediate complement activation to cause vascular leakage	Thayan et al. (2009)
		Up-regulation of alpha1-antitrypsin	Plays role in host immune system	
		Up-regulation of clusterin	Involve in disease severity of dengue virus infection	Ray et al. (2012)
		Up-regulation of alpha1-antitrypsin and alpha1-antichymotrypsin	Play role in host defense mechanism	
		Down-regulation of haptoglobin	Affects free haemoglobin level by forming haptoglobin-haemoglobin complex	

		Up-regulation of vascular cell adhesion molecule-1 (VCAM1), microfibril-associated glycoprotein 4 (MFAP4)	Serve as potential biomarkers to distinguish dengue fever from dengue hemorrhagic fever	Han et al. (2019)
		Up-regulation of fibrinogen-like protein 1 (FGL1)	Can act as a potential biomarker for prediction of dengue hemorrhagic fever development in dengue fever patients	
		Up-regulation of tissue-type plasminogen activator (PLAT) and coagulation factor IX (F9)	Act as markers for identification of dengue hemorrhagic fever	
5	Chikungunya virus	Up-regulation of ras-related protein Rab-8B (RAB8B), dynein heavy chain 5 (DNAH5), dynein heavy chain 12 (DNAH12), vacuolar protein-sorting associated protein 13D (VPS13D) and NSFL1 cofactor 47 (NSFL1C)	May facilitate intracellular virus movement and egression of virus from host cells	Sukkaew et al. (2020)
		Up-regulation of tumor necrosis factor receptor-associated factor 3 (TRAF3)-interacting protein 1	May play role in immune evasion mechanism	
		Up-regulation of copine 9, gephyrine and Rho GTPase-activating protein 5 after 12 h of infection whereas down regulation of same proteins after 24 h of infection	Thought to be involved during early stages of virus infection	
6	Epstein-barr virus	Down-regulation of proteocadherin gamma C3	May have role in natural killer ligand activation	Ersing et al. (2017)
		Down-regulation of viral nuclear antigen 3C, nuclear antigen 2TF and nuclear antigen leader protein	Reveals temporal analysis of several proteins having role during viral infection	
		Down-regulation of Rab7a	Causes alterations in endosomal vesicle trafficking, autophagosome-to-lysosome flux	DeKroon et al. (2018)
		Down-regulation of DnaJ homology subfamily C member 7 (DNAJC7), proteasome subunit beta type-8, ubiquitin specific peptidase 5, ubiquitin specific protease 14 Up-regulation of proteasome subunit beta type-1 (PSMB1), proteasome non-ATPase regulatory subunit 2 (PSMD2), E3 ubiquitin ligase	Role in protein ubiquitination pathway that was identified as one of the top canonical pathways during virus infection	
		Down-regulation of Rab1, Rab38, vacuolar protein-sorting associated protein 24 (VPS24), vacuolar protein-sorting associated protein 37 (VPS37), vacuolar protein-sorting associated protein 4A (VPS24A)	Cause dysregulation of vesicle-trafficking	

(continued)

Table 3.4b (continued)

Serial no.	Virus name	Major protein/s identified	Role of protein/s	References
7	Herpes simplex virus 1	Detection of viral capsid proteins, glycoproteins and tegument proteins.	Provides a comprehensive characterization of virus	Loret et al. (2008)
		Non-detection of unique long region 15/ unique long region 28/ unique long region 33 ($U_L15/U_L28/U_L33$) in mature HSV-1	Suggests either absence of terminase in mature virus particle or dissociation of terminase from viral capsid during some point of time	
		Absence of unique long region 31/ unique long region 34 (U_L31/U_L34) complex	Speculates transitory interactions with viral capsid which do not persist till maturation of virus	
		Identification of one cryptic orphan protein coding sequence piUL49	Activates a viral enzyme necessary for viral replication. Thus, demonstrates neurovirulence property of piUL49	Kato et al. (2020)
		Identification of activator function of piUL49 and regulatory function of Us3 on viral deoxyuridine-triphosphatase.	Suggests important role in viral replication and pathogenesis	
		Degradation of cellular trafficking factor namely Golgi associated PDZ and coiled-coil motif containing protein (GOPC) and reduction of toll-like receptor 2 (TLR2)	Play role in alteration of cellular signaling	Soh et al. (2020)
8	Human cytomegalovirus	Identification of viral proteins such as unique long region 47 (U_L47), unique long region 25 (U_L25), unique long region 88 (U_L88), unique long region 85 (U_L85), unique long region 26 (U_L26), unique long region 48.5 ($U_L48.5$) and one host protein namely immunologically distinct form of actin	Play role in association with purified virus	Baldick and Shenk (1996)
		Identification of viral tegument protein phosphoprotein 65 and glycoprotein M, 12 novel viral proteins and several host cellular structural proteins, enzymes and chaperons.	Provide a picture of comprehensive quantitative analysis of viral and cellular proteins of virus. Together play role in composition of viral particle	Varnum et al. (2004)

		Identification of binding partners for viral proteins unique long region 99 (U_L99) and unique long region 32 (U_L32)	Define two distinct pathways of viral assembly	Moorman et al. (2010)
		Down-regulation of janus kinase 1, signal transducer and activator of transcription 2 and interferon regulatory factor 9	Play role in altersation of interferon signaling pathway	Weekes et al. (2014)
		Identification of myosin XVIII A (MYO18A)	Carries out tethering of viral particles and motor proteins	Jean Beltran et al. (2016)
		Up-regulation of cyclin- dependent kinase 1, cyclin-dependent kinase 7, phosphotidylinositol 3-kinase catalytic subunit type 3 and phosphatidylinositol 4-kinase type 2 beta	Affect several signalling pathways such as adenosine monophosphate-activated protein kinase, mammalian target of rapamycin and extracellular-signal-regulated kinase/ mitogen activated protein kinase	Arend et al. (2017)
		Down-regulation of platelet-derived growth factor receptor alpha, platelet-derived growth factor receptor beta, transforming growth factor beta receptor 1 and transforming growth factor beta receptor 2		
		Virus infection leads to degradation of innate immune system proteins	Proteasome or lysosome carry out such degradation during early stages of infection	Nightingale et al. (2018)
		Enhancement in expression of 60 peroxisomal proteins	Provides importance of peroxisome biogenesis for efficient virus replication	Jean Beltran et al. (2018)

Table 3.4c List of identified proteins using proteomics approaches in various zoonotic virus infection studies along with their role in viral infections

Serial no.	Virus	Major protein/s identified	Role of protein/s	References
1	Rabies virus	Down-regulation of Ca^{2+} ATPase	Cause alterations in ion homeostasis that may lead to neuronal excitation and seizures	Dhingra et al. (2007)
		Up-regulation of Na^{+}/K^{+} ATPase and H^{+} ATPase		
		Down-regulation of collapsing response mediator protein 2 (CRMP2) in spinal cord but up regulation in brain stem	May serve as biomarker for understanding rabies pathogenesis	Thanomsridetchai et al. (2011)
		Up-regulation of glial fibrillary acidic protein (GFAP), tubulin alpha-1 isoform 9, vinculin and xin acting-binding repeat containing 2 isoform 1	Suggests role in host protective mechanism	
			Compensation of damage is not possible due to higher expression of less number of cytoskeletal proteins	
		Down-regulation of dynein light chain, centractin beta isoform, tubulin alpha-1C chain (TUBA1C)	Play role in axonal trafficking and microtubular instability of neurons	Farahtaj et al. (2013)
		Reduction in fatty acid binding protein (FABP3), macrophage migration inhibitory factor (MIF), glutamine synthetase (GS)	Affect neuronal metabolism required for efficient neuronal function	
		Up-regulation of enolase 1 (ENO1)	Affects enzymes of glycolytic activity with an impact on brain metabolism	
		Down-regulation of programmed cell death 6 interacting protein (PDCD6IP)	Leads to neuronal dysfunction	Venugopal et al. (2013)
		Up-regulation of calcium-calmodulin protein kinase II A (CAMK2A)	Shows importance in neuronal signaling	
		Up-regulation of glutamate ammonia ligase (GLUL)	Plays role in host response to viral infection	
		Up-regulation of proteins of oxidative phosphorylation such as ATP5L, ATP5J2, ATP6V1E1, NADH:ubiquinone oxidoreductase core subunit S3, NADH:ubiquinone oxidoreductase subunit B4, NADH:ubiquinone oxidoreductase subunit AB, and ubiquinol-cytochrome c reductase, complex III subunit X	Useful for viral replication, retrograde axonal transport. Also plays role in mitochondrial dysfunction and glycolysis	
		Down-regulation of DEAD- box helicase 3 X-linked (DDX3X)	May play role in virus replication	Behera et al. (2020)
		Up-regulation of DLA-64	Suggests role of host immune response against virus infection	

2	Ebola virus	Identification of heat shock protein A5 (HSPA5)	Shows impactful effect on ebola virus biology	Spurgers et al. (2010)
		Up-regulation of leucine-rich alpha-2-glycoprotein 1 (LRG1)	Emphasizes both severity and degree of inflammation	Ward et al. (2019)
		Up-regulation of Ras-guanosine triphosphatase accelerating protein Src homology 3 domain (Ras-GAP SH3)	May promote host defense mechanism	
		Identification of ribosomal protein L18, ribosomal protein L5, ribosomal protein L3, and ribosomal protein S6	Can provide insight into virus biology	
3	Marburg virus	Non-detection of heat shock protein A5, any ribosomal proteins, actin and tumor susceptibility gene 101 protein	Provides a plausible study for viral infection. However, further investigations are needed	Spurgers et al. (2010)
4	Severe acute respiratory syndrome corona virus 2	Increase production of host translation machinery components	Reshapes host translation process	Bojkova et al. (2020)
		High expression of components of translation, glycolysis, splicing, proteostasis and nucleotide biosynthesis	Can enable determination of potential drug targets against viral infection	
		Higher expression of proteins of neutrophil activation, blood coagulation	Suggests role of proteins in peripheral blood mononuclear cells during viral infection	Li et al. (2020)
		Lower expression of T cell receptor signaling protein		
		Identification of interaction between nuclear factor-kappa-B repressing factor (NKRF) and viral non structural protein nsp10	May shape host immune signature during viral infection	
		Identification of association between tyrosine-protein kinase KIT and open reading frame	May lead to regulation of interferon and interleukin-6 signaling pathways	
		Modulation of mammalian target of rapamycin signalling (mTOR), hypoxia-inducible factor 1 (HIF-1) signaling, tumor necrosis factor (TNF) signaling	Can serve as potential therapeutic target against viral infection	Appelberg et al. (2020)
		Identification of unique peptides from viral nucleoprotein	Can be helpful in development of diagnostic tools for viral infection	Ihling et al. (2020)
		Confident identification of viral nucleoprotein	Helpful in virus detection even at lower viral loads	Nikolaev et al. (2020)

(continued)

Table 3.4c (continued)

Serial no.	Virus	Major protein/s identified	Role of protein/s	References
		Down-regulation of apolipoproteins such as APOA1, APOA2, APOH, APOL1, APOD and APOM	May affect macrophage associated functions	Shen et al. (2020)
		Increased expression of 21-hydroxypregnenolone	Serves a protective mechanism against viral infection	
		Alterations in neutrophil and platelet degranulation and immune system response	Elicitation of host innate immune response and cytokine production	Akgun et al. (2020)

play an essential role in virus replication by interaction with viral proteins (Sun et al. 2008).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) plays role in both carbohydrate metabolism and cellular antioxidative stress. GAPDH undergoes inactivation with a response to various oxidant treatments (Ralser et al. 2007). Following classical swine fever virus infection, down-regulation of GAPDH is suspected to facilitate a metabolic switch to reroute carbohydrate flux as well as to counteract oxidative stress (Sun et al. 2008).

In another study, alterations in proteome profile of peripheral blood mononuclear cells (PBMCs) from classical swine fever virus-infected pigs were also revealed by 2D-PAGE with MALDI-TOF/MS. Out of sixty-six differentially expressed protein spots between infected and uninfected PBMCs, forty-four spots were unambiguously identified as thirty-four unique proteins whereas twenty-two protein spots were unable to detect due to the presence of low quantity. Translation and processing of protein, energy metabolism, antioxidative stress, heat shock proteins and blood clotting proteins were identified host proteins undergoing alterations due to classical swine fever virus infection. Such changes in host's protein profile provide an insight into pathogenic mechanisms of leukopenia and immunosuppression that are clinical symptoms of classical swine fever virus infection (Sun et al. 2010).

Classical swine fever virus infection has led to alterations in proteins of actin cytoskeleton (Sun et al. 2010). Cofilin is a key protein in the regulation of actin dynamics whereas moesin acts as a signal transducer for cytoskeletal remodeling (Bukrinsky 2008; Naghavi et al. 2007). Moreover, an association of vinculin and talin1 proteins causes attachment between microfilament bundles and plasma membrane (Burridge and Mangeat 1984). Alterations in cytoskeletal proteins also lead to apoptotic death (White et al. 2001). Thus, changes in expression level of cytoskeletal proteins due to classical swine fever virus infection may alter cytoskeletal network along with induction of apoptosis (Sun et al. 2010).

Peroxiredoxin-1 (Prx-1) is a stress-induced antioxidant enzyme and acts in a protective mechanism against hydrogen peroxide-induced apoptosis (Berggren et al. 2001). Earlier studies with classical swine fever virus infection in (PK-15) cells have reported an increase in expression of peroxiredoxin-6 (Sun et al. 2008) to provide cellular protection against oxidative stress (Manevich and Fisher 2005). Following classical swine fever virus infection in peripheral blood mononuclear cells, a decrease in expression of Prx-1 might low cellular resistance against oxidative stress and facilitate apoptosis (Sun et al. 2010).

Serum consists of several proteins secreted by cells or tissues. Also, cells or tissues produce proteins which also become part of serum (Kennedy 2002; Sasaki et al. 2002). Studies on serum proteins are highly promising to unravel altered proteomic profiles during any pathophysiological conditions (Issaq et al. 2007). Serum proteomic profiling of pigs with classical swine fever virus infection by 2D-DIGE has found seventeen protein spots in comparison to the serum of uninfected pigs. However, MALDI-TOF/MS could identify ten proteins with down-regulation of six proteins and up-regulation of four proteins in serum of virus-infected pigs (Sun et al. 2011).

Apolipoprotein A-I (apo A-I) contributes to wound recovery by endothelial cell migration into a wound region (Murugesan et al. 1994). Apolipoprotein A-I also inhibits platelet-activating factor synthesis by endothelial cells (Sugatani et al. 1996). Moreover, protection of erythrocytes against procoagulant activity is achieved by apolipoprotein A-I (Epand et al. 1994). In addition, a plasma serine protease namely thrombin plays role in coagulation and hemostasis. Thrombin inhibitor causes inhibition of thrombin and thrombin-induced platelet aggregation, thus, antagonizes host hemostasis. Down-regulation of apolipoprotein A-I and thrombin inhibitor isoform 2 was observed in pig serum post infection with classical swine fever virus. Therefore, disruption of hemostatic balance, a typical symptom of acute classical swine fever is suggested to be a result of a reduction in apolipoprotein A-I and thrombin inhibitor isoform 2 expression (Sun et al. 2011).

Among other down-regulated proteins following classical swine fever virus infection, haptoglobin and vitamin D-binding protein were major ones (Sun et al. 2011). Haptoglobin represents major receptors on leukocytes cell membrane and bears anti-inflammatory activities (El Ghmati et al. 1996). Moreover, in systemic vasculitis patients, haptoglobin serves as an angiogenic factor with a property of angiogenesis stimulation (Cid et al. 1993). Reduction in haptoglobin expression in pigs with classical swine fever virus infection hampers angiogenesis and vascular repair (Sun et al. 2011).

Vitamin D-binding protein causes macrophage activation and chemotaxis of neutrophil (Chishimba et al. 2010). Association with membrane-bound immunoglobulins of B lymphocytes and immunoglobulin G Fc receptor of T lymphocytes is another major function of vitamin D-binding protein. Thus, lower expression of vitamin D-binding protein due to classical swine fever virus infection shows an effect on host immune response and viral pathogenesis (Sun et al. 2011).

Retinol-binding protein 4 (RBP4) remains bind to plasma prealbumins and shows higher expression in plasma of chronic kidney disease patients (Frey et al. 2008). As per mass spectrometry-based proteomics analysis, higher expression of retinol-binding protein 4 in serum of classical swine fever virus-infected pig suggests onset of kidney injury due to viral infection (Sun et al. 2011).

3.4.1.2 Foot-and-Mouth Disease Virus

Foot-and-mouth disease virus (FMDV) is a non-enveloped, positive-strand RNA virus of *Picornaviridae* family and is a causative agent of foot-and-mouth disease which mainly occurs cloven-hoofed animals. The mode of transmission of foot-and-mouth disease virus is either by aerosol or by contact with infected animals (Grubman and Baxt 2004). Approximately, seventy species of cloven-hoofed mammals are affected by foot-and-mouth disease. The associated symptoms of foot-and-mouth disease are fever, discharge from nose and tongue and feet lesions (Ye et al. 2013).

Alterations in host cell proteome due to foot-and-mouth disease virus infection were studied by proteomics approach. SILAC with LC-MS/MS approach has

identified several cellular proteins in porcine kidney cells upon infection with foot-and-mouth disease virus. Porcine kidney (IBRS-2) cells grown in a conventional source of amino acids were designated as mock-infected cells whereas heavy-labeled cells after infection with foot-and-mouth disease virus were designated as infected cells. Following mass spectrometry analysis, one hundred twenty-seven cellular proteins showed differential expression, among them, fifty proteins were down-regulated and seventy-seven proteins were up-regulated in infected IBRS-2 cells (Ye et al. 2013).

Cathepsin D is an aspartyl endopeptidase that activates specific T cells through generation of peptide epitopes in major histocompatibility complex II-mediated pathway (Turk et al. 2002). Upon viral infection, activation of inflammasome and apoptosis occurs by secretion of mature cathepsin D (Rintahaka et al. 2011). In addition, release of apoptosis-inducing factors from mitochondria is also triggered by cathepsin D (Bidère et al. 2003). Thus, cathepsin D is an essential component in normal functioning of immune system and induction of apoptosis. Downregulation of cathepsin D upon foot-and-mouth disease virus infection indicates improper functioning of immune system as well as blockage of apoptosis (Ye et al. 2013).

One of a proteolytic target of cathepsin D is cystatin C. Cystatins protect several endogenous proteases from lysosome. During herpes simplex virus infection, cystatin C inhibits both viral replication and apoptosis induction by cathepsin B (Peri et al. 2007). Furthermore, cystatin C carries out maturation of dendritic cell and induces of tumor necrosis factor (Vray et al. 2002). Higher expression of cystatin C also plays role in host defense mechanism during foot-and-mouth disease virus infection. However, contrasting levels of expression in cathepsin D and cystatin C needs further studies to unravel correlation among them in foot-and-mouth disease virus infection (Ye et al. 2013).

Another proteomics study also utilized SILAC in conjunction with LC-MS/MS approach to study protein alterations during of foot-and-mouth disease virus infection. Infection of baby hamster kidney 21 (BHK-21) cells with foot-and-mouth disease virus lead to a significant alteration in expression level of one hundred fifty-three cellular proteins as compared to uninfected cells. After 6 h of virus infection, ninety-six proteins were down-regulated and fifty-seven cellular proteins were up-regulated (Guo et al. 2015a).

Protein kinase R is an ubiquitously expressed serine and threonine protein kinase. Protein kinase R is also an interferon-induced antiviral and antitumor protein (Balachandran and Barber 2007). Previous reports have revealed that protein kinase R blocks viral replication at protein translation level due to foot-and-mouth disease virus infection. Studies in porcine and bovine cells with inhibitors of protein kinase R have shown an increase in several-fold of virus yield (Chinsangaram et al. 2001). Moreover, knockdown of protein kinase R has not affected wild-type foot-and-mouth disease virus yield (de Los Santos et al. 2006). Foot-and-mouth disease virus infected BHK-21 cells has down-regulated protein kinase R. Cumulative information from earlier reports, lower expression of protein kinase R shows an antagonistic relationship between protein kinase R and foot-and-mouth virus (Guo et al. 2015a).

BHK-21 cells upon foot-and-mouth disease virus infection have down-regulated vacuolar protein sorting-associated protein 28 (VPS28). Also, knockdown of VPS28 gene has increased virus titer (Guo et al. 2015a). As per earlier research, mammalian tumor susceptibility gene-one hundred one (TSG101) binds with VPS28 to form endosomal sorting complex required for transport- I complex. So, VPS28 plays role in virus infection (Bishop and Woodman 2001). Lower expression of VPS28 post foot-and-mouth disease virus infection may function to suppress viral infection (Guo et al. 2015a).

Aminocyl-tRNA synthetases (ARSs) transfer accurate information from genetic code for correct expression of proteins (Woese et al. 2000). Aspartyl-tRNA synthetase is a class II aminoacyl-tRNA synthetase that may lead to progressive cerebellar ataxia, spasticity and dorsal column dysfunction upon mutations in aspartyl-tRNA synthetase encoding gene (Lin et al. 2010; Messmer et al. 2011). Up-regulation of aspartyl-tRNA synthetase was observed in foot-and-mouth disease virus infected BHK-21 cells. In support of RNA interference studies with aspartyl-tRNA synthetase-encoding gene, highly expressed aspartyl-tRNA synthetase may have a role in protein translation of foot-and-mouth disease virus (Guo et al. 2015a). Moreover, aspartyl-tRNA synthetase gene is also known as a calcium-regulated tRNA synthetase to regulate translation machinery in a calcium-dependent manner (Jaiswal and Nanjundiah 2003). Therefore, following foot-and-mouth disease virus infection, increased intracellular calcium levels may lead to higher expression of aspartyl-tRNA synthetase (Guo et al. 2015a).

3.4.1.3 Bovine Herpesvirus 1

Bovine herpesvirus 1 (BHV-1) is a causative agent for bovine rhinotracheitis, a contagious and infectious disease of cattle. Bovine herpesvirus 1 consists of a double-stranded DNA genome and belong to *Varicellovirus* genus of *Herpesviridae* family (Muylkens et al. 2007; Nandi et al. 2009). Infected animals sometimes undergo secondary bacterial infections to cause shipping fever. Infection with bovine herpesvirus 1 also leads to conjunctivitis, reproductive tract lesions, encephalitis, fetal infections and latency in natural hosts (Jones and Chowdhury 2007).

Differential proteomic analysis of bovine herpesvirus 1 infected madin-darby bovine kidney (MDBK) cells was carried out to distinguish effect of two viral strains. A proteomics approach 2D-PAGE along with MALDI-TOF/MS has examined the differential protein expression of eight proteins between infected and uninfected cells concerning virulent and attenuated bovine herpesvirus 1 infection. The identified eight proteins were pyruvate kinase, heat shock protein 90 AA1, heat shock protein 90 AB1, annexin A, scinderin, tubulin (alpha 1a), vimentin and albumin. Only four proteins namely pyruvate kinase, tubulin, vimentin and heat shock protein 90 AB1 were found in a virulent strain of bovine herpesvirus 1. Identified proteins were thought to be involved in cell migration, tumor formation and development, cell invasion and viral infection and tumor cell line apoptosis (Guo et al. 2015b).

Heat shock protein 90 AB1 (HSP90AB1) was found only in cells with virulent strain infection (Guo et al. 2015b). According to earlier reports, heat shock protein 90 AB1 plays role in cell migration, tumor formation and development, cell invasion, viral infection and tumor cell line apoptosis (Coskunpinar et al. 2014; Ernst et al. 2014; Haase et al. 2014; Huang et al. 2014b). However, in bovine herpes virus infection, heat shock protein 90 AB1 has been recently identified but the role of this protein during viral infection is not yet known (Guo et al. 2015b). Therefore, investigations are still ongoing to find out role of heat shock protein AB1 during virus infection.

Another heat shock protein 90 AA1 (HSP90AA1) is a molecular chaperone and involves in cell regulation, maintenance of cellular proteins conformation to carry out cell survival under various stimulation (Trepel et al. 2010; Yang et al. 2013). Proteomics analysis could identify heat shock protein 90 AA1 in control cells and cells after infection with an attenuated strain of bovine herpesvirus 1 (Guo et al. 2015b). However, further studies are needed to understand role of heat shock protein 90 AA1 in molecular mechanisms behind bovine herpesvirus 1 pathogenesis.

A comparative proteome study of extracellular bovine herpesvirus 1 and herpes simplex virus 1 virion was carried out using 1D-PAGE with nanoscale liquid chromatography-mass spectrometry (nanoLC-MS/MS) to identify both host and virus proteins. Human keratinocyte cells (HaCaT) were infected with herpes simplex virus 1 whereas bovine herpesvirus 1 was infected in MDBK cells. Bovine herpesvirus 1 has identified eight hundred eighty-three host proteins whereas four hundred eighty-eight host proteins were found in herpes simplex virus 1 infection. Moreover, fifty-one virus proteins were identified out of which only nine proteins were unique to bovine herpesvirus 1 and forty-two proteins were common between herpes simplex virus 1 and bovine herpesvirus 1. Identification of forty-two viral proteins in both viruses was an indication of protein conservation due to genome homology. During infection with several herpes viruses, non-infectious light particles (L-particles) are also produce along with progeny viruses. Non-infectious light particles lack viral capsid but contain an enveloped tegument structure (Alemañ et al. 2003; Carpenter et al. 2008; Dargan et al. 1995; Ibiricu et al. 2013; McLauchlan and Rixon 1992; Szilágyi and Cunningham 1991). According to proteomics analysis, light particles of bovine herpesvirus 1 and herpes simplex virus 1 bear a similar complement of envelope proteins and differ in composition of tegument proteins (Russell et al. 2018).

Compared to herpes simplex virus 1, light particles from bovine herpesvirus 1 have shown depletion of three major tegument proteins namely unique long region 36 (U_L36), unique long region 37 (U_L37) and unique long region 48 (U_L48). Moreover, three other tegument proteins such as U_L21 and U_L16 were also depleted in bovine herpesvirus 1 light particles (Russell et al. 2018). In several herpes viruses, U_L36, U_L37 and U_L48 create a link between viral tegument and capsid or envelope proteins (Owen et al. 2015). As per previous studies, U_L21 plays role in nuclear egress of herpes simplex virus 1 and herpes simplex virus 2 capsids (Le Sage et al. 2013; Sarfo et al. 2017). U_L16 is essential for nuclear egress of only herpes simplex virus 2 and affects virus replication (Gao et al. 2017; Starkey et al. 2014). Such

findings from proteomics analysis suggest a role of several proteins of bovine herpesvirus 1 light particles in bridging of capsid and envelope as well as in viral replication (Robinson et al. 2008; Russell et al. 2018).

A recent study by 2D-PAGE proteomics approach with MALDI-TOF/TOF has found several proteins during initial phase of bovine herpesvirus 1 infection. Ribonuclease/angiogenin inhibitor 1 (RNH1) was down-regulated in MDBK cells during bovine herpesvirus 1 infection (Magalhães-Junior et al. 2020). Ribonuclease angiogenin, a homolog of human angiogenin, stimulates transcription of stress-induced small RNAs derived from transfer RNA as well as cleaves transfer RNA during cellular stress conditions. In contrast, transcription of stress-induced small RNAs derived from transfer RNA and transfer RNA cleavage is inhibited by RNH1 during bovine herpesvirus 1 infection (Li and Hu 2012). Decreased expression of RNH1 may play role in host defense mechanism during bovine herpesvirus 1 infection.

Another novel discovery during initial phase of bovine herpesvirus 1 infection was identification of Rab guanosine diphosphate dissociation inhibitor alpha 2 (GDI2). Rab guanosine diphosphate dissociation inhibitors (GDIs) regulates vesicular membrane traffic. Therefore, down-regulation of GDI2 was found to have some role due to bovine herpesvirus 1 infection (Magalhães-Junior et al. 2020).

3.4.1.4 Murine Herpesvirus

Murine herpesviruses (MHVs) are double-stranded DNA virus of *Herpesviridae* family (Blaskovic et al. 1980; Rajcáni et al. 1985). Macrophages, B lymphocytes, lung alveolar cells and endothelial cells are target sites of murine herpesvirus infection. Murine herpes virus can lead to life-long latency in host B lymphocytes and macrophages (Rajćáni and Kúdelová 2005). Murine herpesviruses have been widely used in animal models as well as in several host cells to study biology of viruses (Flaño et al. 2000; François et al. 2010; Peng et al. 2010).

Functional roles of conserved virion proteins have been carried out with a mass-spectrometry based proteomics approach. Proteome studies of purified murine gammaherpesvirus 68 (MHV68) from infected 293T or NIH3T3 cells by 1D-PAGE with microLC-MS/MS have identified fourteen viral structural proteins. Name of few identified proteins was open reading frame 75c (ORF75c) encoded tegument protein, five nucleocapsid protein from ORF26, ORF62, ORF25, ORF65/M9, ORF29 and two envelope glycoproteins namely glycoprotein B and glycoprotein H. In addition, virion-associated proteins were encoded from ORF20, ORF24, ORF28, ORF45, ORF48 and ORF52 were also found through proteomics approach (Bortz et al. 2003). Identified proteins may involve in initial phase of viral infection or viral assembly and egress of virus.

Further, another murine herpesvirus namely murid herpesvirus-4 was studied using 1D-PAGE with nanoLC-MS/MS proteomics approach for identification of murid herpesvirus-4 structural proteins. As a result, thirty-one viral structural proteins were identified with thirteen tegument proteins, eight capsid proteins, nine

envelope proteins whereas one protein remains as an unclassified structural protein. Thirteen tegument proteins were encoded by ORF11, ORF21, ORF23, ORF33, ORF36, ORF38, ORF45, ORF52, ORF55, ORF63, ORF64, ORF75c, ORF75b. Identification of eight capsid proteins was expressed from ORF25, ORF26, ORF19, ORF32, ORF62, ORF43, ORF65 and ORF17. Moreover, nine envelope proteins of virus were encoded from ORF4, ORF8, ORF22, ORF27, ORF28, ORF39, ORF47, ORF51 and ORF58. All the identified proteins provide insight into structural components of murine herpesvirus (Vidick et al. 2013).

Parallelly, murid herpesvirus 4 infection also detected several host proteins including annexin A2. However, no role of annexin A2 upon viral growth was depicted during *in vitro* studies. Overall, identified viral and host proteins were highly beneficial to understand the biology of murine herpesvirus infection (Vidick et al. 2013).

3.4.1.5 Murine Cytomegalovirus

Murine cytomegalovirus (MCMV) is another cytomegalovirus that shares 45.2% sequence identity with human cytomegalovirus. Murine cytomegalovirus belongs to *Herpesviridae* family and consists of double-stranded DNA genome. Murine cytomegalovirus can infect epithelial cells, endothelial cells, fibrocytes, myocytes, brown fat adipocytes, macrophages and bone marrow stromal cells (Krmpotic et al. 2003). Diseases due to cytomegalovirus infection have also been studied with murine cytomegalovirus infection (Rawlinson et al. 1996). Species-specificity of human cytomegalovirus to only human hosts has emphasized use of murine cytomegalovirus as a small animal model of human cytomegalovirus. Conserved genome organization along with tissue tropism are key factors for increasing importance of murine cytomegalovirus studies (Krmpotic et al. 2003; Rawlinson et al. 1996). Functional investigations of murine cytomegalovirus conserved genes would be beneficial for understanding life cycle of human cytomegalovirus.

Proteomics study with purified virions from infected mouse embryonic fibroblasts (MEFs) using 1D-PAGE with nanoLC-MS/MS have determined protein composition of several murine cytomegaloviruses. Proteomics research has identified thirty-eight proteins of virus capsid, virus tegument, glycoprotein and belong to virus replication and immunomodulatory protein families. Out of thirty-eight proteins, one tegument protein M25 and major capsid protein M86 were the most abundant proteins found to be involved in complex composition of murine cytomegalovirus (Kattenhorn et al. 2004).

3.4.1.6 Pseudorabies Virus

Pseudorabies virus (PRV) is a member of *Varicellovirus* genus *of Herpesviridae* family. Structure of complex multilayered extracellular virion consisting of both viral and host proteins is conserved among other herpesviruses (Pomeranz et al.

2005). Pseudorabies virus is a neuroinvasive virus and is used for studying mammalian neuronal circuits as well as herpesvirus entry and morphogenesis (Ekstrand et al. 2008; Mettenleiter 2002, 2004).

A proteomics approach, 2D-PAGE with peptide mass fingerprint analysis was used for analysis of purified pseudorabies virion components and to find out multiple isoforms of several viral as well as host-derived proteins. Glycosylation isoforms of pseudorabies virus glycoprotein B and multiple isoforms of major capsid protein larger unique long 19 were successfully detected in purified virions from infected porcine kidney cells (Michael et al. 2006). Moreover, annexin A1, A2 and heat shock protein 70 were identified as structural components of purified pseudorabies virus. (Michael et al. 2006). Human cytomegalovirus (Varnum et al. 2004), rabies virus (Sagara and Kawai 1992) and primate lentiviruses (Gurer et al. 2002) have been reported with heat shock protein 70 as a structural component of viral particle. Several isoforms of heat shock protein 70 were also identified in pseudorabies virus-infected porcine kidney cells by 2D-PAGE (Michael et al. 2006).

Furthermore, SILAC in combination with peptide mass fingerprint analysis mass spectrometry has been used for quantitation of pseudorabies virus structural components between wild type and mutant pseudorabies viruses (Michael et al. 2006). Studies with stable isotope mass tags have shown pseudorabies virus without one tegument protein unique short 3 (Us3) causes a reduction in incorporation of another tegument protein unique long region 46 (U_L46). Such observation was previously reported in herpes simplex virus 2 (Matsuzaki et al. 2005). In contrast, deletion of Us3 and other tegument proteins such as U_L47 or U_L49 in pseudorabies virus has not affected relative amount of U_L38 capsid protein, U_L25 capsid-associated protein, U_L36 tegument proteins and glycoprotein H. Moreover, deletion of pseudorabies virus tegument proteins Us3, U_L47 or U_L49 has resulted in incorporation of actin in pseudorabies virus. More incorporation of actin was observed during the absence of U_L47 protein in comparison to absence of Us3 and U_L49 protein. Thus, either lack or absence of tegument proteins Us3, U_L47 or U_L49 suggested tegument replenishment by cellular actin in pseudorabies virus (Michael et al. 2006).

Two complementary mass spectrometric approaches have been used to characterize protein content of pseudorabies virus in purified virions from infected PK-15 cells (Kramer et al. 2011). First, in-gel digestion of proteins in 1D-PAGE and analysis on orbitrap MS/MS (Luo et al. 2010) was performed for rapid detection of pseudorabies virus proteins. However, to avoid sample loss due to in-gel digestion, a second modified proteomics approach with in-solution digestion of samples prior to nanoLC-MS/MS analysis (Wiśniewski et al. 2009) was followed for pseudorabies virus protein characterization. In addition to identification of thirty-five viral proteins by proteomics approaches, in-solution digestion approach could identify twelve additional viral proteins making it more suitable for characterization of complex viral particles. Among forty-seven viral proteins, seven viral proteins namely U_L8, U_L20, U_L32, U_L40, U_L42, U_L50, and Rsp40/infected-cell protein 22 were identified as novel pseudo rabies virus proteins. Moreover, one of these novel viral protein Rsp40/infected-cell protein 22 was found to be an orthologue of infected-cell protein 22 (ICP22) of herpes simplex virus 1 (Kramer et al. 2011). In herpes

simplex virus, ICP22 modulates viral gene expression (Bowman et al. 2009) and maintains proper virus morphology (Orlando et al. 2006). ICP22 also induces discrete nuclear foci formation with cellular chaperone proteins (Bastian et al. 2010), deciphering potential role of Rsp40/infected-cell protein 22 during pseudorabies virus infection (Kramer et al. 2011).

Key modification like phosphorylation, one type of post-translational modification serves dynamic functions during viral infections (Edson 1993; Morrison et al. 1998; Stevely et al. 1985). Identification of four phosphorylated viral proteins namely U_L26, U_L36, U_L46, and U_L48 in pseudorabies virus infection suggests their abundance in viral particles. According to earlier findings, orthologues of respective proteins in herpes simplex virus 1 infection undergo phosphorylation after viral entry (Morrison et al. 1998). The presence of phosphorylated proteins on extracellular pseudorabies virus particle speculates role of phosphorylation in viral proteins activation during early stages of infection (Kramer et al. 2011).

Pseudorabies virus-Bartha (PRV-Bartha) is an attenuated strain that induces a substantial reduction of an inflammatory response in comparison to highly virulent pseudorabies virus strains (Ekstrand et al. 2008). Bartha strain of pseudorabies virus serves as a vaccine strain to study molecular mechanisms behind pathogenesis in neurons due to retrograde spreading and attenuated virulence (Lomniczi et al. 1987). After pseudorabies virus infection in C57/BL mice, a quantitative proteomics approach with LC-MS/MS was used to study host response along with interaction between virus and host neurons. Proteomics study with infected mouse brain tissue has identified greater than two thousand proteins at tissue level and greater than one thousand three hundred proteins at synaptosome level. Furthermore, comparative proteome profiling between virulent and attenuated pseudorabies virus has revealed co-regulation among several host proteins of synaptic transmission and cytoskeleton organization having roles in virus trafficking (Zeng et al. 2018).

ADP-ribosylation factor-like protein 3 (Arl3) is a guanosine triphosphatases (GTPase) family member related to adenosine diphosphate (ADP)-ribosylation factors and is necessary for trans-golgi network (Graham 2004). During pseudorabies virus infection, Arl3 showed around 1.7 fold expression in synaptosomes. Furthermore, involvement of trans-golgi network was thought to have a role in virus sorting to axonal compartment as well as in intracellular spread from axons (Zeng et al. 2018). Synaptic transmission-related proteins like shank protein family promote maturation and enlargement of dendritic spines (Sheng and Kim 2000). In addition, a synaptosomal-associated protein receptor protein called snap29 plays role in synaptic transmission (Su et al. 2001). Lower expression of a shank protein family and snap29 during both virulent and attenuated rabies virus infection suggests inhibition of host synaptic transmission during pseudorabies virus infection (Zeng et al. 2018).

3.4.1.7 Porcine Circovirus

Porcine circoviruses (PCV) are *Circoviridae* family members having single-stranded circular genome. Porcine circovirus type 1, porcine circovirus type 2 and porcine circovirus type 3 are three genotypes of porcine circovirus (Ouyang et al. 2019). Porcine circovirus type 2 is a novel porcine circovirus associate with several diseases in pigs (Allan and Ellis 2000). Every swine-producing country bears porcine circovirus type 2 infection which is gradually increasing year after year (Gillespie et al. 2009). Porcine circovirus disease is caused by porcine circovirus type 2 infection with symptoms like weight loss, pneumonia, enlarged lymph nodes, reproductive failure, vasculitis and necrotizing dermatitis (Cheng et al. 2011; Lin et al. 2011; Madec et al. 2008; Madson et al. 2009).

A proteomics approach SILAC with LC-MS/MS has identified one hundred sixty-three significantly altered proteins upon porcine circovirus type 2 infection in PK-15 cells in comparison to uninfected cells. Among one hundred sixty-three proteins, eighty-four proteins showed lower expression whereas seventy-nine proteins had higher expression in virus-infected cells. PK-15 cells with porcine circovirus type 2 infection have shown twenty-eight fold increase in a calcium-dependent non-lysosomal protease, calpain-1 (Fan et al. 2012). Hepatitis C virus as well as hepatitis B virus infection have shown up-regulation of calpain protein. In hepatitis C virus infection, calpain activation causes inhibition of extrinsic apoptotic signaling pathway (Simonin et al. 2009) whereas, in hepatitis B virus infection, calpain significantly induces migration of liver cells (Zhang et al. 2010). Calpain proteases activate by ryanodine receptor to promote synaptic dysfunction as per earlier reports in human immunodeficiency virus type 1 infection (Perry et al. 2010). Fortunately, porcine circovirus type 2 infection in PK-15 cells has increased seven fold expression level of ryanodine receptor 1 (Fan et al. 2012). Therefore, higher expression of both calpain and ryanodine receptor 1 in porcine circovirus infection suggests an involvement of these two proteins in viral pathogenesis.

Several cytoskeletal proteins undergo down-regulation following porcine circovirus type 2 infection (Fan et al. 2012). Previous studies regarding cytoskeletal disruption have shown dispersion of vimentin and beta-tubulin networks are crucial for the release of viral particles from infected cells (Chen et al. 2008; Fang et al. 2009; Pocernich et al. 2005). Therefore, the role of cytoskeletal proteins is speculated to have a role in mechanism of porcine circovirus type 2 pathogenesis.

To investigate host and viral proteins interaction, co-immunoprecipitation along with LC-MS/MS analysis in infected PK-15 cells has identified two hundred twenty-two host proteins in interaction with viral capsid protein (Zhou et al. 2020). As circovirus capsid protein together with replicase protein regulates virus replication (Finsterbusch et al. 2009; Timmusk et al. 2006), the interaction between capsid protein of virus and several host proteins would be helpful to study several stages of virus life cycle inside host body. Proteomics analysis found an interaction of eight proteins of host such as heterogeneous nuclear ribonucleoprotein C, nucleophosmin1, DEAD-box RNA helicase 21, importin beta3, eukaryotic translation initiation factor 4A2, MX dynamin like GTPase 2, snail family transcriptional repressor

2, and intermediate chain 1 with viral capsid protein (Zhou et al. 2020). Validation of such proteins by co-immunoprecipitation and pull-down assays has provided insight into viral replication and pathogenesis.

Another type of porcine circovirus called porcine circovirus type 3 causes porcine dermatitis, neuropathy syndrome, reproductive failure in piglets and sows. iTRAQ followed LC-MS/MS analysis in caudal left lung lobe from infected piglets has shown alterations in two hundred forty-two cellular proteins after 3 weeks of porcine circovirus type 3 infection as compared to uninfected piglets. Among two hundred forty-two differentially expressed proteins, one hundred forty-two proteins were down-regulated whereas one hundred proteins were up-regulated. Metabolic processes, innate immune response, phagosome pathways were deduced as major functions of differentially regulated proteins due to porcine circovirus type 3 infection (Jiang et al. 2020).

Apart from its role in ATP production, ATP synthase has a key role in binding, assembly, maturation and budding processes of many viruses (Ahmad et al. 2013). In human immunodeficiency virus infection, viral transfer between antigen-presenting cells and $CD4^+$ cells is mediated by ATP synthase (Yavlovich et al. 2012). As in porcine circovirus type 2, replication is an energy-dependent process, up-regulation of ATP synthase upon porcine circovirus type 3 infection is a pre-requisite for viral self-proliferation depending upon host metabolic processes (Jiang et al. 2020).

Type I interferons and pro-inflammatory cytokines are components of innate immune response for host defense against viral infections. Porcine circovirus type 2 infection causes inhibition of innate immune response proteins such as interferon and suppression of interferon regulatory factor 3 and interferon-beta promoters (Li et al. 2018). Contrastingly, porcine circovirus type 3 infection causes up-regulation of interferon-induced proteins. Up-regulation of immune response proteins suggests activation of an interferon signaling pathway to contribute resistance against porcine circovirus type 3 infection (Jiang et al. 2020).

Moreover, modulation of both innate and acquired immune responses is another result of phagocytosis of pathogens (Jaumouillé and Grinstein 2016). Significant up-regulation of proteins like iC3b, collectins, p40phox, major histocompatibility class-I and class-II upon porcine circovirus infection (Jiang et al. 2020), is evidence of phagosome pathway activation as observed in earlier studies (Kudo et al. 2004; Mantegazza et al. 2013; Suh et al. 2006; Yuste et al. 2008).

3.4.2 Human Viruses

3.4.2.1 Influenza Virus

Influenza virus (IFV) is *Orthomyxoviridae* family member. Influenza viruses are small enveloped viruses with eight segments of single negative-strand RNA genome. However, influenza virus B is clinically relevant for humans. Cellular response to

influenza virus infection has been widely studied by transcriptomic analysis (Baas et al. 2006; Geiss et al. 2002; Hutchinson 2018). However, as messenger RNA (mRNA) levels do not provide sufficient information, alterations in cellular proteins due to influenza virus infection were characterized by several proteomics studies.

Proteomics analysis with influenza A virus infected in madin-darby canine kidney (MDCK) and human lung carcinoma cell line (A549) has revealed the effect of virus at cellular proteome level. Analysis of 2D gels has identified sixteen differentially expressed protein spots in both infected mammalian cell lines in comparison to uninfected cells. 2D-DIGE coupled with nanoLC-MS/MS analysis have identified cytoskeletal protein keratin 10 and interferon-induced key components like Mx proteins (Vester et al. 2009). Keratin 10 may facilitate transport of progeny virus where Mx proteins may play role in signal transduction and apoptosis during influenza A virus infection. Therefore, identified proteins were involved either in life cycle of virus or host cell stress response. Thus, proteomics studies have revealed host-virus interactions to emphasize antiviral research in support with vaccine manufacture (Vester et al. 2009).

A proteomics approach SILAC along with 2D-PAGE combined with LC-MS/MS was used to study cellular proteome in influenza virus infected human lung A549 cells and has found differential expression of two hundred eighty proteins at a 95% confidence limit in comparison to uninfected cells. Out of two hundred eighty proteins, one hundred twenty-seven proteins were up-regulated with involvement in stress responses, regulation of mRNA transcription, initiation of translation and major histocompatibility complex I dependent immunity pathways. Similarly, one hundred fifty-three proteins were down-regulated with a role in alternative splicing, major histocompatibility complex II-dependent immunity, nucleic acid metabolism, adhesion and cytoskeleton regulation (Coombs et al. 2010).

Beta-catenin, a target of ubiquitin-proteasome pathways, plays role in regulation of human lung development (Aberle et al. 1997; De Langhe et al. 2008). Down-regulation of beta-catenin by influenza virus infection is suspected to hamper interferon induction which is essential for an antiviral response (Coombs et al. 2010; Shapira et al. 2009). Moreover, WD40 is a beta-catenin binding protein showed approximately hundred-fold down-regulation in influenza virus infected human lung cells (Coombs et al. 2010). Being involved in retrovirus insertion (Jiang et al. 2002) and herpes virus replication (Smith et al. 2000), lower level expression of WD40 due to influenza virus infection is suspected to hamper several signal transduction pathways as well as molecular binding processes.

Influenza virus is dependent upon host polymerase II transcripts to favor the viral replication process as well as for priming viral transcription process (Engelhardt et al. 2005). Following influenza virus infection, general transcription factor IIA undergoes approximately four-fold down-regulation. Transcription factor IIA plays role in the regulation of RNA polymerase II-dependent DNA transcription (Coombs et al. 2010; Høiby et al. 2007). Being an RNA virus, even though influenza virus does not require any DNA intermediate for replication, down-regulation of transcription factor IIA dependent host transcription would be important for interferon and interferon-inducible genes.

Upon influenza virus infection in human cells, higher expression of major histocompatibility complex I cause immune evasion by its incorporation into ganglioside-rich microdomains. As a result, inhibitors of natural killer cell binding and function are recruited to facilitate immune evasion (Culley 2009). A decrease in expression level of several components of major histocompatibility complex I due to influenza virus infection is suspected to decrease viral antigen presentation on cell surface. As a result, immune cell-mediated attack occurs (Coombs et al. 2010).

In recent studies, comparative pulse-labeling experiments by a combination of bioorthogonal amino acid such as azidohomoalanine (AHA) and SILAC was carried out in A549 cells infected with two influenza virus strains such as human-adapted and bird-adapted strain. After digestion, analysis of peptide samples by proteomics approach has identified seven thousand seven hundred twenty-nine host proteins and ten viral proteins in infected A549 cells. Moreover, quantification of six thousand twenty-nine proteins was successfully carried out in biological replicates. Differences in host and viral protein expression observed as several orders of magnitude during 8–12 h pulse interval with induction of viral proteins and gradual reduction of host proteins over time. Such level of expression for viral and host proteins was similar for both human-adapted and bird-adapted strains (Bogdanow et al. 2019).

According to previous reports, reduced expression of host proteins known as host shutoff is facilitated by influenza A virus through a reduction in host mRNA levels. Suppression of host cellular mRNA translation, as well as simultaneous enhancement of viral mRNA translation, was found to be a major reason behind viral-induced host shutoff (Bercovich-Kinori et al. 2016). Therefore, a decline in host protein expression, particularly during 8–12 h of infection, showed a correlation with a reduction in host mRNA levels in influenza virus-infected cells. But such correlation between mRNA and protein level expression was more for bird-adapted influenza virus strain in comparison to human-adapted influenza virus strain. Post-transcriptional modifications are suspected to be the reason behind such variability for different virus strains (Bogdanow et al. 2019).

The proteomic analysis also revealed role of viral proteins during permissive and non-permissive infection. In contrast to host proteins, inefficient production of viral matrix protein 1 was observed during non- permissive infection with bird-adapted strain. Increased splicing of viral M segment RNA is responsible for incompetent viral matrix protein 1 production. 3′ splice site region of viral RNA shows important signatures of mammalian adaptation (Reid et al. 2002). Therefore, RNA splicing of viral M segment is modulated by 3' splice site which may be a probable reason behind the 1918 pandemic of influenza virus (Bogdanow et al. 2019).

In case of farm animals, cattles are known as the reservoir species of a novel influenza virus called influenza D virus. As per the evidences, this virus causes bovine respiratory disease (Ferguson et al. 2016) and remains associate with respiratory system in feedlot cattle (Ng et al. 2015). A study in Mississippi beef cattle has shown young, weaned, comingled and immunologically naive calves were most susceptible to infection because of the dwindling maternal antibody levels after 6 months of age (Ferguson et al. 2015), however, the influenza D virus pathogenesis

in cattle is not well understood. Additionally, studies in United States has confirmed presence of Influenza C virus in cattle with clinical signs of bovine respiratory disease (Zhang et al. 2018), however, role of this virus needs detailed investigations.

3.4.2.2 Human Immunodeficiency Virus

Human immunodeficiency virus is a *Retroviridae* family member (Nájera et al. 1987). Studies on epidemiologic and phylogenetic analysis have revealed introduction of human immunodeficiency virus into the human population during 1920–1940. Human immunodeficiency virus type 1 has originated from central African chimpanzees and human immunodeficiency virus type 2 has evolved from west African sooty mangabeys. However, human immunodeficiency virus type 1 is more common. (Faria et al. 2014; Gao et al. 1999; Sharp and Hahn 2011). Genome of human immunodeficiency virus type 1 consists of two identical single-stranded RNA molecules. Early period of human immunodeficiency virus type 1 infection shows unspecific symptoms such as diarrhea, malaise, fatigue and weight loss. Gradual viral infection may lead to a weak immune response, and development of neoplasms (Iwuji et al. 2013; Mocroft et al. 1996).

Human immunodeficiency virus type 1 progresses replication cycle inside a host by degradation of many cellular pathways. Moreover, several host cellular factors facilitate human immunodeficiency virus assembly and budding (Demirov and Freed 2004; Freed 2002; Marsh and Thali 2003). During human immunodeficiency virus type-1 infection in purified virions from human monocyte-derived macrophages (MDMs), two hundred fifty-three proteins were successfully identified using LC-MS/MS proteomics approach. Among two hundred fifty-three proteins, two hundred twenty proteins were reported for the first time in human immunodeficiency virus type 1 particles including highly dominated cytoskeleton, metabolic, ubiquitin/proteasomal, adhesion, signaling, intracellular trafficking, and immune response system proteins. Identified cellular proteins can serve as potential targets for therapeutic studies in human immunodeficiency virus type 1 infection (Chertova et al. 2006).

It is well known that cluster of differentiation 4 (CD4) T cells are targets of human immunodeficiency virus type 1. CD4 T cells remain profoundly involved in human immunodeficiency virus life cycle at several interfaces of host-virus interactions. Moreover, several proteins of HIV cause CD4 cell apoptosis and subsequent death (Bartz and Emerman 1999; Cottrez et al. 1997). Therefore, even after the discovery of drugs against human immunodeficiency virus proteins, several cellular cofactors still serve as targets for novel therapeutics.

Alterations in cellular proteome of primary CD4 cells after human immunodeficiency virus type 1 infection were analyzed through LC-MS/MS approach. At 24 h post-viral infection, one hundred sixty-eight cellular proteins were reported with a change in abundance in comparison to uninfected cells. Moreover, as observed with annexin V staining and flow cytometry analysis, 24 h post-viral infection is the time point where apoptosis is unnoticeable in CD4 cells (Chan et al. 2009). Therefore,

detection of a change in cellular proteome at 24-h post-viral infection suggests a dynamic correlation of host proteins with human immunodeficiency virus type 1 infection.

Replication of human immunodeficiency virus is dependent upon both cellular and viral components. G-rich sequence factor 1 is a host mRNA-binding protein that plays role in nuclear localization of spliced transcripts of human immunodeficiency virus type 1 (Jablonski and Caputi 2009). Following 8 h of post-infection, proteomics studies have found up-regulation of G-rich sequence factor 1 (GRSF1) in infected CD4 cells. Increased expression of such ribonucleoprotein G-rich sequence factor 1 also maintains stability of viral transcript (Chan et al. 2009). Furthermore, according to earlier studies, host cytoskeletal proteins such as integrin and transgelin play role in uncoating of internalized human immunodeficiency virus (Lafrenie et al. 1996; Shrikant et al. 1996). As identified by proteomics studies with LC-MS/MS approach, higher expression of integrin and transgelin due to HIV-1 infection in CD4 cells are thought to be useful in facilitation of virus replication cycle (Chan et al. 2009). Thus, alterations in cellular factors can be targeted to fight against human immunodeficiency virus infection.

Nullbasic is a mutated human immunodeficiency virus type 1 with replacement of viral trans-activator of transcription (Tat) protein basic domain with glycine and arginine residues (Apolloni et al. 2013). Studies have shown an interaction between viral proteins and cellular proteins is helpful for transport of viral mRNA from nucleus to cytoplasm (Naji et al. 2012; Suhasini and Reddy 2009). To find an interaction between nullbasic and cellular proteins, LC-MS/MS-based proteomics approach has identified forty-six host proteins in virus-infected HEK293T cells in comparison to uninfected cells (Lin et al. 2014). Among forty-six host proteins, B23 and chromosomal maintenance 1 protein were reported in the functional regulation of a viral protein namely regulator of viral protein expression (Rev). B23 is a nucleolar protein known to have a role in nuclear import of Rev (Szebeni et al. 1997).

Similarly, during nuclear export, chromosomal maintenance 1 (CRM1) protein helps for Rev protein interaction with nuclear pore complex (Neville et al. 1997). Rev of human immunodeficiency virus type 1 binds to Rev Response Element (RRE) of unspliced viral mRNAs. Rev protein also facilitates nuclear export of such mRNAs in CRM1 receptor pathway (Naji et al. 2012). Moreover, with respect to previous reports, sub cellular localization of B23 and CRM1 can be altered by nullbasic in Rev protein dependent manner (Lin et al. 2012).

DEAD (aspartate-glutamate-alanine-aspartate) box helicases are largest RNA helicase family and found conserved among archaea, bacteria and eukaryotes (Linder and Jankowsky 2011). Several cellular DEAD box helicases are previously reported to have key roles during human immunodeficiency virus type 1 replication (Lorgeoux et al. 2012). Earlier, mass spectrometry analysis detected forty-six nullbasic-binding proteins, out of which, three were DEAD-box RNA helicases namely DEAD box helicase 1 (DDX1), DEAD box helicase 3 (DDX3) and DEAD box helicase 17 (DDX17). After identification of (DDX1) by proteomics studies, co-immunoprecipitation experiments have shown an interaction between nullbasic and endogenous DDX1 (Lin et al. 2014). As per earlier findings, human

immunodeficiency virus type 1 infection causes overexpression of DDX1 increases virus production. At same time, down-regulation of DDX1 by RNA interference inhibits virus replication.

Localization of Rev protein expression protein from nucleolus to either nucleus or cytoplasm has been observed in cells and astrocytes upon human immunodeficiency virus type 1 infection. Alteration in subcellular localization of Rev was observed with respect to a decrease in expression of DDX1 (Edgcomb et al. 2012; Fang et al. 2004, 2005). Thus, DDX1 has some role in subcellular localization of human immunodeficiency virus type 1 Rev protein.

Complexity associated with the life cycle of human immunodeficiency virus type 1 is a big challenge to study molecular mechanisms of viral infection. Human immunodeficiency virus type 1 utilizes membrane proteins for entry and cellular proteins for transport, assembly and maturation. Moreover, viral transcription is dependent upon nuclear factors whereas several regulatory mechanisms are associated with maintenance of viral life cycle (Donnelly and Ciborowski 2016). Proteomic profiling techniques offer in-depth knowledge of host-virus interaction that may help to investigate human immunodeficiency virus type 1 pathology.

Farm animals such as cattle and buffalo are susceptible to persistent infection with bovine immunodeficiency virus and remain associate with symptoms like lymphadenopathy, lymphocytosis, lesions in central nervous system that lead to gradual weakness (Carpenter et al. 1992; Van der Maaten et al. 1972). Moreover, decrease in milk yield (McNab et al. 1994), reduction in lymphocytic blastogenic response (Martin et al. 1991) and bovine paraplegic syndrome (Walder et al. 1995) are some other symptoms of cattle. Despite its close genetic and antigenic similarity with human immunodeficiency virus, bovine immunodeficiency virus is considered as non-pathogenic and its infection in cattle health is not clearly established (Bhatia et al. 2013).

3.4.2.3 Japanese Encephalitis Virus

Japanese encephalitis virus (JEV) belongs to *Flaviviridae* family with single positive-strand RNA genome. Mosquitoes are vectors of japanese encephalitis virus that definitely vary according to different geographical regions but the major vector is *Culex* mosquito (Solomon 2006). Infection with respect to human infection, pigs are major contributors of virus transmission cycle (Ghosh and Basu 2009). Japanese encephalitis virus infection leads to symptoms like fever, headache, meningeal irritation and alteration in consciousness. Neuronal death due to severe infection leads to a high mortality rate whereas neuronal squeal occurs in some patients who survive (Kumar et al. 1990).

A proteomics approach 2D-PAGE with mass spectrometry approach has identified fifty-six differentially expressed proteins in neuronal cells (Neuro2A) following japanese encephalitis virus infection when compared to uninfected cells. Heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) has been observed to assist hepatitis C virus replication (Lee et al. 2011). Another member of same

family known as heterogeneous nuclear ribonucleoprotein A2 has shown to participate in japanese encephalitis virus replication (Katoh et al. 2011). Proteomics analysis by 2D-PAGE with MALDI-TOF/TOF has revealed up-regulation of HNRNPH1 in japanese encephalitis virus-infected mouse neuronal cells. Enhanced expression of HNRNPH1 suggests virus-induced invasion of host machinery for successful viral replication (Sengupta et al. 2014).

Earlier, a proteomics study with LC-MS/MS approach in infected and uninfected neuroblastoma SK-N-SH cells of human has shown role of a 57 kilodalton intermediate filament protein vimentin in virulence of Japanese encephalitis virus infection (Liang et al. 2011). Moreover, in infected and uninfected stable porcine kidney cells, MALDI-TOF/MS has identified a 60 kilodalton protein molecule namely vimentin as a possible receptor for japanese encephalitis virus (Das et al. 2011). Microtubular rearrangement and redistribution are other major alterations of host proteome that occur by japanese encephalitis virus infection (Chiou et al. 2003). Cytoskeletal perturbations in Japanese encephalitis virus-infected mouse neuronal cells is thought to be helpful for viral protein transport to convoluted membranes which act as viral protein reservoir during multiplication of virus (Sengupta et al. 2014).

Endoplasmic reticulum serves as an essential organ for stress response induced by misfolded proteins of cell (He 2006). Unfolded protein response is cellular machinery to respond to cellular stress response by inducing molecular chaperones and by degrading and clearance of unfolded proteins (Schröder and Kaufman 2005). Neuro2A cells, after infection with japanese encephalitis virus have revealed up-regulation of a host stress response protein ERP29 and down-regulation of a protein disulfide isomerase family protein ERP57 by proteomics studies (Sengupta et al. 2014). During unfolded protein response due to japanese encephalitis virus infection, high-level expression of ERP29 would enhance host stress response as observed earlier by an association of ERP29 with endoplasmic reticulum chaperone BiP (Mkrtchian et al. 1998). Similarly, ERP57 participates in plasma membrane signaling events and nuclear signaling events (Turano et al. 2011). Therefore, a decrease in ERP57 expression would affect such signaling pathways during viral infection.

In *in vitro* studies, japanese encephalitis virus-infected HeLa cells have identified one hundred fifty-eight differential expressed proteins in comparison to uninfected cells by SILAC along with LC-MS/MS proteomics approach. Some of induced proteins were part of interferon-induced groups such as Interferon-stimulated gene 15, GTPase, interferon-induced GTP-binding protein Mx1, protein kinase R, 2′-5′-oligoadenylate synthetase 3 (Zhang et al. 2013). Interferon-stimulated genes are host innate immune response components and act in defense against viral infections (Sadler and Williams 2008). However, antiviral potentials of several interferon-stimulated genes are still not properly known. Interferon-induced transmembrane protein 3 (IFITM3) is known to inhibit human immunodeficiency type I virus infections through interference with viral entry and replication (Lu et al. 2011). Moreover, interferon-induced transmembrane protein 3 acts as an inhibitor of hepatitis C virus translation process (Yao et al. 2011). So, up-regulation of Interferon-induced

transmembrane protein 3 in infected cells is expected to influence japanese encephalitis virus infection (Zhang et al. 2013).

Another interferon-stimulated gene product known as sterile alpha motif domain-containing protein 9 (SAMD9) showed a higher level of expression in japanese encephalitis virus-infected cells (Zhang et al. 2013). As per earlier observations with sendai virus and poxvirus infection, up-regulation of SAMD9 may play role in host's antiviral effect against virus infection. (Liu et al. 2011; Tanaka et al. 2010).

Apart from interferon-stimulated genes, vesicle-associated membrane proteins have also shown antiviral effects upon virus infection. Following japanese encephalitis virus infection, vesicle-associated membrane protein 8 (VAMP8) was up-regulated. VAMP8 causes transport vesicles fusion to cognate membranes (Wong et al. 1998). In japanese encephalitis virus-infected cells, depletion of VAMP8 expression by small interference RNA (siRNA) technology has shown increased virus infection. Thus, VAMP8 is suggested to act as an antiviral factor for japanese encephalitis virus infection (Zhang et al. 2013).

As a whole, japanese encephalitis virus infection is coordinated through several host responses which can be potential targets for antiviral therapy (Zhang et al. 2013).

In cattle, japanese encephalitis virus can occasionally cause febrile illness and encephalitis in cattle and study with japanese encephalitis virus infected 114-month-old cow was identified with the viral antigen. Moreover, the virus was isolated from the infected cerebrum and has shown close relationship with G1 isolates of pigs and mosquitoes (Kako et al. 2014). Early signs of depression, loss of appetite and neurological impairments are some of the clinical features associated with japanese encephalitis virus infection in cattle (Kako et al. 2014; Katayama et al. 2013). Regardless of these findings, onset of japanese encephalitis virus infection in cattle is not completely known and this needs further study to identify the actual causes of the virus infection.

3.4.2.4 Dengue Virus

Dengue virus (DENV), member of *Flaviviridae* family has single positive-strand RNA genome. Dengue fever is the characteristic symptom of dengue virus infection and severe forms of symptoms include dengue hemorrhagic fever and dengue shock syndrome (Guha-Sapir and Schimmer 2005). If not treated at early stages, dengue virus infection may lead to life-threatening complications like vascular permeability, hemorrhagic manifestations and thrombocytopenia (Cardier et al. 2005).

A 2D-PAGE long with MALDI-TOF/MS has found an elevated level of dengue virus non-structural protein 1 in serum of dengue hemorrhagic fever patients in comparison to dengue fever patients (Thayan et al. 2009). Up-regulation of viral non-structural protein 1 also leads to complement activation, a key feature of vascular leakage during dengue hemorrhagic fever (Avirutnan et al. 2006). Viral non-structural protein 1 is expressed at the cell surface and acts as a target of human responses (Jacobs et al. 2000). Therefore, upon dengue virus infection, up-regulation of non-structural protein 1 remains associate with disease severity. In addition,

dengue hemorrhagic fever patients have also shown higher expression of a 50 kilodalton serine protease inhibitor namely alpha1- antitrypsin (Thayan et al. 2009). Alpha1-antitrypsin is crucial for maintaining homeostasis between protease-antiprotease and to prevent against proteolytic tissue damage (Bristow et al. 1998). In macrophages and neutrophils, the activity of proteases is mediated by inhibitory action of alpha1-antitrypsin (Ren et al. 2004). Thus, the role of host immune system is highlighted during disease severity with dengue virus infection (Thayan et al. 2009).

A proteomics approach 2D-DIGE with MALDI-TOF/MS has identified significant changes in the expression level of forty-eight proteins in serum of dengue fever patients in comparison to healthy controls. Serum proteomics study found twenty-five proteins with lower expression and twenty-three proteins with higher expression (Ray et al. 2012). Higher expression of clusterin, a complement inhibitory factor was found in serum of dengue virus-infected patients (Ray et al. 2012). Earlier studies have shown an association between host clusterin protein and dengue virus non-structural protein 1 are responsible for plasma leakage which is a symptom of dengue virus infection (Kurosu et al. 2007). So, up-regulation of clusterin is thought to involve in disease severity of dengue virus infection.

Haptoglobin is an acute-phase protein found with a lower expression level in serum of dengue fever patients (Ray et al. 2012). Anti-inflammatory activity and innate phenotype-dependent antioxidant activity are associated functions of haptoglobin. During hemolysis, haptoglobin removes free hemoglobin and plays an important role during angiogenesis (Quaye 2008). Low-level haptoglobin expression in dengue fever patients' sera reduces free hemoglobin by formation of the haptoglobin-haemoglobin complex. Later on, macrophages and monocytes clear such complexes in serum of dengue fever patients (Ray et al. 2012).

Proteomics studies have found higher-level expression of several serine protease inhibitors such as alpha1-antitrypsin and alpha1-antichymotrypsin in dengue fever patients' sera (Ray et al. 2012). Up-regulation of serine protease inhibitors is thought to serve as a response of host against dengue virus infection in support of their similar expression in dengue fever as well as dengue hemorrhagic fever patients (Albuquerque et al. 2009; Thayan et al. 2009). In addition to dengue virus infection, overexpression of alpha1-antitrypsin and alpha1-antichymotrypsin was observed in severe acute respiratory syndrome-coronavirus infection (Wan et al. 2006) and hepatitis B virus infection (Tan et al. 2011). Therefore, several serine protease inhibitors play role in the host defense mechanism against viral infections.

Dengue fever can develop into dengue hemorrhagic fever with similar clinical characteristics (Calderón-Peláez et al. 2019; Halstead 2019). Even though pathogen and early clinical symptoms are same for both types of diseases, understanding of gradual development of dengue hemorrhagic fever in dengue fever patients is still a challenge. To identify potential molecular biomarkers of dengue hemorrhagic fever development in dengue fever patients, TMT based nanoLC-MS/MS proteomics approach was carried out in dengue fever patients before the onset of dengue hemorrhagic fever and compared with healthy controls. In total, four hundred forty-one

proteins were differentially expressed in serum of dengue fever patients (Han et al. 2019).

TMT-based proteomics approach has identified up-regulation of few proteins namely vascular cell adhesion molecule-1, microfibril-associated glycoprotein 4 and fibrinogen-like protein 1 in dengue fever patients. Expression of these three proteins was also significantly increased in dengue hemorrhagic patients (Han et al. 2019). Vascular cell adhesion molecule-1 (VCAM1) has a regulatory effect on inflammation-associated vascular adhesion. The progression of several immunological disorders is also associated with VCAM1 (Kong et al. 2018). Earlier research on dengue virus infection has shown elevation in plasma concentrations of vascular cell adhesion molecule-1 (Koraka et al. 2004; Murgue et al. 2001). Microfibril-associated glycoprotein 4 (MFAP4) is characterized as a fibrillin-binding protein with a role in cell adhesion or intercellular interactions mediated by calcium (Pilecki et al. 2016). Increased expression of VCAM1 and MFAP4 in serum of dengue fever patients showed even higher level expression in dengue hemorrhagic fever patients (Han et al. 2019). Thus, VCAM1 and MFAP4 can serve as potential biomarkers to distinguish dengue fever from dengue hemorrhagic fever.

Activation of antigen-specific T cells can be inhibited by fibrinogen-like protein 1 (FGL1) (Wang et al. 2019). In contrast to the expression of other proteins, level of FGL1 expression was increased in dengue fever and dengue hemorrhagic fever patients. Different levels of FGL1 expression in different dengue patients offer an opportunity for the virus to escape host's immune response. Therefore, fibrinogen-like protein 1 can be utilized as a biomarker for prediction of dengue hemorrhagic fever development in dengue fever patients (Han et al. 2019).

Higher expression of proteins like tissue-type plasminogen activator (PLAT) and coagulation factor IX (F9) was observed particularly in dengue hemorrhagic patients (Han et al. 2019). PLAT converts plasminogen to plasmin and plasmin is responsible for degradation of fibrin with an increased risk of bleeding. F9 activates coagulation factor X to participate in coagulation process at site of bleeding. Due to specific higher-level expression in dengue hemorrhagic fever patients, PLAT and F9 can be utilized as markers of dengue hemorrhagic fever identification (Han et al. 2019).

In farm animals including cattle, dengue virus infection is not yet reported. Hence, no information is available about dengue virus infection in cattle.

3.4.3 Chikungunya Virus

Chikungunya virus (CHIKV) is a positive-strand RNA virus of *Togaviridae* family (Caglioti et al. 2013). Chikungunya virus infection causes chikungunya fever (CHIKF) with symptoms like high fever, nausea, polyarthralgia or arthritis (Borgherini et al. 2008; Schilte et al. 2013; Singh and Unni 2011). In humans, chikungunya fever occurs by *Aedes aegypti* mosquito bite in which persistence of virus for long period of time leads to severe arthritis in minor joints (Paixão et al. 2018).

Chikungunya virus infected human fibroblast-like synoviocytes (HFLS) cells have shown alterations in many host proteins in comparison to uninfected cells. A gel-enhanced liquid chromatography-mass spectrometry (Ge-LC-MS/MS) proteomics approach has identified differential expression of two hundred fifty-nine proteins after 12 h and two hundred forty-one proteins after 24 h of chikungunya virus infection (Sukkaew et al. 2020).

Earlier reports with west nile virus infection have shown a role of ras-related protein (Rab-8B) in virus transport to the plasma membrane (Kobayashi et al. 2016). In case of human immunodeficiency virus type 1 and herpes simplex virus infection, host's dynein system has played a major role in intracellular transportation (Döhner et al. 2002; Lehmann et al. 2009). Children with severe dengue virus infection have shown involvement of vacuolar protein sorting-associated protein 13D (VPS13D) in extracellular vesicular exosomal transport (Nhi et al. 2016). Moreover, another protein namely NSFL1 cofactor 47 (NSFL1C) plays role in vesicle transport and membrane fusion (Kondo et al. 1997). Reduction in NSFL1C has reduced infection of west nile virus (Krishnan et al. 2008). Several other proteins such as ras-related protein Rab-8B (RAB8B), VPS13D, dynein heavy chain 5 (DNAH5), dynein heavy chain 12 (DNAH12) and NSFL1C have shown higher expression in chikungunya virus-infected human fibroblast-like synoviocytes. Enhanced expressions of several host proteins are thought to facilitate intracellular movement of virus particles as well as egression of chikungunya virus from host cells (Sukkaew et al. 2020).

From cellular defense machinery, chikungunya virus infection leads to up-regulation of tumor necrosis factor receptor-associated factor 3 (TRAF3)-interacting protein 1 (Sukkaew et al. 2020). As per earlier reports of TRAF3-interacting protein 1 with a negative role in type I interferon production (Ng et al. 2011), immune evasion mechanism is thought to be an associated function of TRAF3-interacting protein 1 after chikungunya virus infection (Sukkaew et al. 2020).

Based on time point study, several proteins such as a membrane trafficking protein copine 9, cytoskeleton organization protein gephyrine, Rho GTPase-activating protein 5 showed higher expression after 12 h of infection. Same proteins undergo down-regulation after 24 h post chikungunya virus infection (Sukkaew et al. 2020). Role of such proteins is thought to be involved during early stages of virus infection.

A study has shown chikungunya virus isolation in cattle of central Africa, however, these animals may play a negligible role in the virus life cycle (Guilherme et al. 1996). Furthermore, chikungunya virus infection in farm animals may vary among different animals and regions around the globe. However, no further reports are available.

3.4.3.1 Epstein-Barr Virus

Epstein-Barr virus (EBV) is a member of *Herpesviridae* family (Johannsen et al. 2004). Genome of epstein-barr virus is composed of double-stranded DNA. Epstein-barr virus is an etiologic agent of Hodgkin disease, nasopharyngeal carcinoma,

burkitt's lymphoma and malignant lymphomas (Cohen et al. 2011; Raab-Traub 2007). Contact with oropharyngeal secretions containing epstein-barr virus is a major mode of transmission (Newcom 2001; Young and Rickinson 2004). In addition, blood and blood derivative transfusions, organ and tissue transplantation are several other modes of epstein-barr virus transmission (Cohen et al. 2009).

TMT-based orbitrap MS/MS approach for proteomics analysis in infected and uninfected human Burkitt lymphoma B cells during epstein-barr virus replication has revealed sixty-nine viral encoded proteins. Among several pathways, B-cell receptor signaling pathway was highly significant among down-regulated proteins. Also, complement pathways along with blood coagulation pathways were highly enriched among up-regulated proteins. An early expressed epstein-barr virus protein targets both cell surface and intracellular B-cell receptor complex during lytic replication for ubiquitin-mediated proteasomal degradation. Lytic B cells produce immunoglobulins to trap epstein-barr virus during infection. But B-cell receptor complex degradation enables epstein-barr virus to get avoided by immunoglobulin binding either in secretory pathway or cell surface manner (Ersing et al. 2017).

Furthermore, tonsillar plasma cells, a hub of immunoglobulins, are thought to be the site of epstein-barr virus lytic replication. Approximately, 200 molecules of immunoglobulins are secreted by plasma cells per second (Hibi and Dosch 1986). Therefore, degradation of immunoglobulins by targeting B-cell receptor complex is hypothesized to stop both immunoglobulin secretion and production of infectious epstein-barr virus (Ersing et al. 2017).

In addition to host defense mechanisms, host's complement components regulate of T-cellular metabolism and homeostatic survival (Hess and Kemper 2016). Up-regulation of complement pathways after epstein-barr virus infection needs more studies to decipher involvement of complement system in host defense mechanism or in activation of metabolic and cell survival pathways (Ersing et al. 2017).

Several antiviral innate immune proteins are targets for multiple viruses (Schoggins et al. 2011). Combining proteomics data of epstein-barr virus replication with temporal human cytomegalovirus infection or with kaposi'ssarcoma-associated herpes virus have identified down-regulation of ten host proteins by these three herpes viruses infection. Down-regulation of protocadherin gammaC3 by epstein-barr virus and cytomegalovirus suggests role of protocadherin gamma C3 in natural killer ligand activation as earlier reported for protocadherin FAT1 (Ersing et al. 2017; Timms et al. 2013; Weekes et al. 2014). Quantification by TMT-based proteomics approach resulted BMRF1, BLRF2, and BALF2 were most abundantly expressed epstein-barr virus proteins whereas epstein-barr virus nuclear antigen 3C, epstein-barr virus nuclear antigen 2TF and epstein-barr virus nuclear antigen leader protein were three least expressed proteins. Moreover, proteomics study has shown a thousand times greater concentration of most abundant protein BMRF1 in comparison to least expressive epstein-barr virus nuclear antigen 3C protein. Thus, quantitation of approximately 80% of epstein-barr virus proteins by proteomics approach has revealed a temporal analysis of several protein expressions (Ersing et al. 2017).

Two oncoproteins of epstein-barr virus namely latent membrane protein 1 (LMP1) and latent membrane protein 2A (LMP2A) affect cellular ubiquitination by interaction with cellular ubiquitin ligases. LC-MS/MS proteomics approach between infected and uninfected MCF10A breast epithelial cells has analyzed effect of LMP1 and LMP2A on global cellular proteome and has identified more than seven thousand seven hundred proteins (DeKroon et al. 2018). As per previous studies, during latency, blockage of autophagic flux occurs by reactivation of epstein-barr virus (Granato et al. 2014). Recently, proteomics study has stated expression of Rab7a host protein undergo a reduction in presence of both LMP1 and LMP2A. Decrease in Rab7a expression alters both endosomal vesicle trafficking as well as autophagosome-to-lysosome flux. Moreover, several regulatory pathways like cell death and survival, actin filament dynamics and cell movement were also found to be shared by both LMP1 and LMP2A. Besides, epstein-barr virus proteins target proteasome subunits, ubiquitin-specific conjugases and peptidases and vesicle-trafficking proteins (DeKroon et al. 2018).

Alterations in expression of proteins such as DnaJ homology subfamily C member 7 (DNAJC7), 26S proteasome non-ATPase regulatory subunit 2 (PSMD2), 26S proteasome non-ATPase regulatory subunit 8, proteasome subunit beta type-1 (PSMB1), proteasome subunit beta type-8 (PSMB8), ubiquitin-specific peptidase 5, ubiquitin-specific protease 14 and E3 ubiquitin ligase suggests protein ubiqutination pathway to be most affected by infection with epstein-barr virus. Furthermore, following epstein-barr virus infection, several vacuolar ATPase subunits and exocyst subunits such as Rab1, Rab38, vacuolar protein sorting-associated protein 24 (VPS24), vacuolar protein sorting-associated protein 37 (VPS37) and vacuolar protein sorting-associated protein 4A (VPS4A) undergo down regulation. A decrease in expression of such vesicle-trafficking proteins suggests epstein-barr virus infection leads to dysregulation of vesicle trafficking. Thus, proteomics analysis and follow-up studies have found a role of LMP1 and LMP2A on ubiquitination and alterations of cellular proteins to be responsible for cell growth regulation (DeKroon et al. 2018).

In case of farm animals, epstein-barr virus infection not yet reported and no more related information available at present.

3.4.3.2 Herpes Simplex Virus 1

Herpes simplex virus 1 (HSV-1) belongs to *Herpesviridae* family. Pandemics of various herpes diseases have been caused by both herpes simplex virus (Xu et al. 2019). Herpes simplex virus 1 has a double-stranded linear DNA genome. Herpes simplex virus 1 causes orofacial infections and encephalitis. Establishment of latent infections in sensory neurons is property of herpes viruses which may cause lesions at a later stage (Whitley and Roizman 2001).

Comprehensive protein analysis of herpes simplex virus 1 (HSV-1) using nanoLC-MS approach has revealed forty-eight viral proteins and forty-nine distinct host proteins after infection in HeLa cells. Among forty-eight proteins of herpes simplex virus 1, eight viral capsid proteins, thirteen viral glycoproteins,

twenty-three viral tegument proteins and four novel viral proteins were identified. Moreover, two tegument proteins namely infected-cell protein 0 (ICP0) and infected-cell protein 4 (ICP4) were detected as extracellular virion components whereas precise localization of four novel viral proteins unique long region 7 (U_L7), unique long region 23 (U_L23), unique long region 50 (U_L50), and unique long region 55 (U_L55) were not properly studied. Among host proteins, several transport, cytoskeletal and enzymatic proteins were found abundantly due to herpes simplex virus 1 infection (Loret et al. 2008). According to earlier findings, unique long region 15/unique long region 28/unique long region 33 ($U_L15/U_L28/U_L33$) terminase is the component of immature herpes simplex virus type 1 capsids and plays role in DNA packaging (Wills et al. 2006). However, non-detection of ($U_L15/U_L28/U_L33$) terminase in mature extracellular herpes simplex virus 1 by mass spectrometry analysis has suggested either absence of viral terminase in mature extracellular virions or its dissociation from capsid during some point of time. Such observation emphasizes a dynamic interaction between virus and terminase (Loret et al. 2008).

Moreover, proteomics study has revealed absence of unique long region 31/unique long region 34 (U_L31/U_L34) complexes on mature extracellular herpes simplex virus 1 in support with previous studies. Absence of U_L31/U_L34 complex speculates transitory interactions with capsids which do not persist till maturation of the virus (Loret et al. 2008; Reynolds et al. 2002).

In a very recent and innovative study, characterization of newly synthesized proteome of herpes simplex virus 1 was carried out by bioorthogonal noncanonical amino acid tagging (BONCAT) based mass spectrometry analysis (Kato et al. 2020). The proteome of a cell consists of both old and newly synthesized proteins. But both old and new proteins become chemically indistinguishable due to sharing of same amino acids pool. Bioorthogonal non-canonical amino acid tagging is an advanced technology to distinguish newly synthesized proteins in a cell by introducing azide groups (Dieterich et al. 2006). Upon herpes simplex virus 1 infection in human SK-N-SH cells, nine cryptic orphan protein-coding sequences were identified by BONCAT based mass spectrometry analysis in comparison to uninfected cells. In-depth characterization of one cryptic orphan protein-coding sequence piUL49 has shown role of piUL49 in activation of a viral enzyme necessary for viral replication (Kato et al. 2020). Viral replication through newly synthesized proteins demonstrates neurovirulence property of cryptic orphan protein-coding sequence in cells infected with herpes simplex virus 1.

In macrophages and neurons, several viruses encode deoxyuridine-triphosphatases (dUTPases) to compensate for lower activity of host deoxyuridine-triphosphatases (Chen et al. 2002; Hizi and Herzig 2015; Payne and Elder 2001). Like some other viruses, herpes simplex virus 1 encodes uracil-DNA glycosylase that functions like viral dUTPase. Function of viral dUTPase is to prevent point mutation during viral replication so that genome integrity of virus does not undergo any change (Chen et al. 2002; Hizi and Herzig 2015; Priet et al. 2006). A characterization study has shown the activator function of piUL49 on viral dUTPase. Moreover, one serine-threonine protein kinase of herpes simplex virus 1 namely Us3 phosphorylates viral dUTPase with a regulatory effect. Thus, both activator function and regulatory

effect on viral dUTPase are important for herpes simplex virus 1 replication and pathogenesis as observed in several earlier studies (Kato et al. 2014, 2015, 2020).

Quantitative temporal viromics is an advanced quantitative analysis to study temporal changes in both host and virus during time course of virus infection (Weekes et al. 2014). Quantitative temporal viromics employs TMTs along with triple-stage mass spectrometry for quantitation of proteins. Human keratinocytes are natural targets of lytic herpes simplex virus 1 infection. Quantitative temporal viromics analysis in herpes simplex virus 1 infected human keratinocytes has identified seven thousand human proteins as well as greater than 90% of canonical viral proteins as compared to uninfected keratinocytes. Among them, degradation of several host proteins namely coiled-coil motif-containing protein (GOPC) and cellular trafficking factor Golgi-associated PDZ. Further studies with plasma membrane profiling have shown a reduction of immune signaling molecule toll-like receptor 2 (TLR2) in plasma membrane of cells after infection with herpes simplex virus 1 (Soh et al. 2020).

Herpes simplex virus-encoded protein unique long region 56 (U_L56) is a tail-anchored type II membrane protein (Koshizuka et al. 2002). GOPC is a binding partner of unique long region 56 and proteomics analysis has shown that ubiquitination of GOPC as well as proteasomal degradation is mediated by U_L56 (Soh et al. 2020). Moreover, the expression of TLR2 is dependent upon GOPC. Therefore, results from quantitative temporal viromics with herpes simplex virus infection state degradation of a cellular trafficking factor GOPC which is responsible for alteration of host protein toll-like receptor 2 on surface of infected cells (Soh et al. 2020).

Herpes simplex virus 1 has similar biological properties with another member of *Herpesviridae* family called bovine herpesvirus 1 which is the causative agent for several diseases in cattle. The detail of bovine herpesvirus 1 is described in the animal virus Sect. 3.4.1.3.

3.4.3.3 Human Cytomegalovirus

Human cytomegalovirus (HCMV) is a *Herpesviridae* family member consists of viral as well as cellular gene products and encodes more than two hundred predicted open reading frames (Varnum et al. 2004). Human cytomegalovirus genome consists of approximately linear double-stranded DNA. About 10% of infants get an infection by transmission of human cytomegalovirus from mother by placenta or by breastfeeding. Human cytomegalovirus is also considered an important pathogen of immunocompromised individuals (Landolfo et al. 2003). Infection with human cytomegalovirus leads to enlargement of cells and formation of inclusion bodies (Schottstedt et al. 2010).

Studies with denaturing 1D-PAGE and subsequent protein sequencing in purified virions isolated from infected primary human foreskin fibroblasts (HFFs) have identified six human cytomegalovirus proteins namely unique long region 47 (UL47), unique long region 25 (UL25), unique long region 88 (UL88), unique long

region 85 (UL85), unique long region 26 (UL26), and unique long region 48.5 (UL48.5) along with a 45 kDa host protein. After sequencing, 45 kilodalton host protein was identified as an immunologically distinct isoform of actin (Baldick and Shenk 1996). Later on, a comprehensive quantitative analysis of viral and cellular proteins of human cytomegalovirus was carried out by a non-gel-based two-stage proteomics approach. LC-MS/MS and fourier transform ion cyclotron resonance (FTIR) mass spectrometry in purified virions from infected human dermal fibroblasts have reported twelve novel human cytomegalovirus open reading frames to encode viral proteins. Along with several host cellular structural proteins, enzymes and chaperons, a tegument protein of virus called phosphoprotein 65 and glycoprotein M together compose infectious human cytomegalovirus particle (Varnum et al. 2004).

A targeted genetic-proteomic approach was utilized in purified recombinant virions from infected primary human foreskin fibroblasts (HFFs) to generate molecular events behind human cytomegalovirus assembly. Analysis of purified recombinant virions from infected HFFs with MALDI linear trap quadrupole (LTQ)-orbitrap cross-linking mass spectrometer has identified binding partners for viral protein unique long region 99 (UL99) and unique long region 32 (UL32). Both proteins play a major role during viral assembly process. UL32 viral protein traffic in a clathrin-associated pathway in association with capsid proteins like unique long region 46 (UL46), unique long region 85 (UL85), and unique long region 80.5 (UL80.5) and several tegument proteins such as unique long region 48 (UL48), terminal repeat sequence 1, unique long region 69 (UL69), unique long region 97 (UL97), unique long region 25 (UL25), and unique short region 22 (US22). On the other hand, during the initial stages of virion assembly, another viral protein namely UL99 carries out an ubiquitin-mediated endosomal sorting complex required for transport (ESCRT) pathway. Thus, during initial stages of human cytomegalovirus infection, two important proteins of viral assembly UL32 and UL99 traffic through two distinct pathways. (Moorman et al. 2010).

In addition, human cytomegalovirus envelope protein glycoprotein B was found to traffic in another distinct pathway with localization in small puncta throughout the cytoplasm. Contrastingly, during later stages of human cytomegalovirus infection three parallel distinct pathways of UL32, UL99 and glycoprotein B merge representing final stage of viral assembly (Moorman et al. 2010).

Quantitative temporal viromics with TMTs has quantified one thousand two hundred cell-surface proteins during human cytomegalovirus infection in primary human fetal foreskin fibroblasts (HFFFs) in comparison to uninfected cells. Interferon signaling pathway proteins such as signal transducer and activator of transcription 2 (STAT2), janus kinase 1 (JAK1) and interferon regulatory factor 9 (IRF9) were down-regulated by infection with human cytomegalovirus merlin strain (Weekes et al. 2014). Using a similar strategy, greater than 250 human proteins were targeted in infected HFFFs as compared to uninfected HFFFs. Among them, proteins of innate immune function undergo degradation by proteasome or lysosome during early stages of human cytomegalovirus infection as observed in primary HFFFs (Nightingale et al. 2018). Another proteomic study with TMTs has shown

changes in temporal and spatial protein expression during human cytomegalovirus infection in primary human fibroblasts. Subcellular organization of four thousand host proteins and remodeling of lysosomes into two distinct subpopulations was observed in primary human fibroblasts due to viral infection (Jean Beltran et al. 2016).

To understand expressional changes in host kinases, another new proteomics approach, a multiplexed kinase inhibitor bead-mass spectrometry (MIB-MS) found temporal alterations in complement of host protein kinases during human cytomegalovirus infection in human MRC-5 fibroblasts as compared to uninfected cells. Infection with human cytomegalovirus in primary human MRC-5 fibroblasts has shown increased expression of cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 7 (CDK7) and several lipid kinases like phosphatidylinositol 3-*kinase* catalytic subunit *type* 3 (PI3KC) and phosphatidylinositol 4-*kinase type* 2 beta (PI4K2B). In contrast, expression of platelet-derived growth factor receptor α (PDGFRα), platelet-derived growth factor receptor β (PDGFRβ) and transforming growth factor beta receptor 1 (TGFBR1) and transforming growth factor beta receptor 2 (TGFBR2) were decreased due to infection with both strains of human cytomegalovirus. Importance of several signaling pathways such as adenosine monophosphate-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR) and extracellular-signal-regulated kinase/ mitogen activated protein kinase (ERK/ MAPK) was deduced to get hampered by human cytomegalovirus infection (Arend et al. 2017).

A targeted proteomics approach during human cytomegalovirus infection in MRC5 primary human fibroblasts has shown global changes in peroxisome biogenesis in comparison to uninfected cells. Analysis of sixty peroxisomal proteins at 0, 6, 24, 48, 72 and 120 h post-infection in MRC5 primary human fibroblasts with parallel reaction monitoring (PRM) assay has revealed enhancement of major peroxisomal proteins from 24 h onwards. Therefore, peroxisome biogenesis was found to be essential for efficient replication of human cytomegalovirus infection (Jean Beltran et al. 2018).

No information or report yet available about human cytomegalovirus infection in farm animals.

3.4.4 Zoonotic Viruses

3.4.4.1 Rabies Virus

Rabies virus (RABV) belongs to *Rhabdoviridae* family of viruses. Rabies virus is a neurotropic virus with a single negative-strand RNA genome. Rabies is a life-threatening disease occurs due to infection with rabies virus. Bats and dogs are major vectors of rabies virus but dog-transmitted rabies poses the greatest hazard all over the world. In India, greater than 95% of reported cases of dog rabies are documented annually (Fooks et al. 2017; Jackson 2002; Mahadevan et al. 2016). Clinical

features of rabies virus infection include 20–90 days of asymptomatic incubation period, 2–10 days of partially symptomatic predromal period and 2–7 days of a severely symptomatic acute neurologic period. Once symptoms appear, an infected animal or person undergoes death within 2–7 days (Jackson 2002).

A proteomics approach 2D-PAGE with MALDI-TOF/MS has found differential expression of more than fifty-five proteins in rabies virus-infected ICR mouse brain tissue in comparison to uninfected mouse. Infection of mouse with silver-haired bat rabies virus decreased expression of an ion homeostasis protein Ca^{2+} ATPase in contrast to overexpression of Na^{+}/K^{+} ATPase and H^{+} ATPase (Dhingra et al. 2007). Alterations in proteins of ion homeostasis may be a reason behind excitation and seizures in neurons (Somjen 2002) as ion homeostasis undergoes disruption more in brain tissue as compared to other tissue (Stys et al. 1992; Urenjak and Obrenovitch 1996).

To extend knowledge of rabies pathogenesis, paralytic and furious dogs were experimented at their proteome level using 2D-PAGE along with quadrupole time-of-flight mass spectrometer (qTOF/MS). Remarkable alterations in proteome profile were observed among infected tissues from brainstem, hippocampus and spinal cord of dogs with paralytic and furious rabies when compared with respective uninfected tissues. Proteomics study has found differential expression of thirty-two proteins in hippocampus, forty-nine proteins in brainstem and sixty-seven proteins in spinal cord among clinical groups. In brainstem and spinal cord of dogs with paralytic rabies, differential expression of antioxidants depicts terminal stage of rabies virus infection (Thanomsridetchai et al. 2011). During both paralytic and furious rabies, most of cytoskeletal proteins undergo down-regulation (Laothamatas et al. 2011) whereas in contrast to previous study, vinculin, glial fibrillary acidic protein (GFAP), tubulin alpha-1 isoform 9 and xin acting-binding repeat-containing 2 isoform 1 were significantly up-regulated in proteomics study of paralytic and furious rabid dog brain tissues. Such contrasting level of cytoskeletal protein expression suggests a host protective mechanism against viral infection through reorganization of cytoskeletal proteins. However, as the number of up-regulated cytoskeletal proteins is less, compensation of central nervous system damage is not possible (Thanomsridetchai et al. 2011).

Collapsing response mediator protein 2 (CRMP2) has been known to have a role in axonal growth and branching (Fukata et al. 2002), neuronal differentiation in hippocampal cultures (Inagaki et al. 2001) and in migration of peripheral T-lymphocytes (Varrin-Doyer et al. 2009). CRMP2 was less expressed in infected spinal cord of both paralytic and furious dogs whereas highly expressed in brainstem of only paralytic dogs (Thanomsridetchai et al. 2011). Such unusual behaviour of CRMP2 may serve as tissue biomarker for a better understanding of rabies pathogenesis.

Proteomics analysis of rabid human brain tissues by 2D-PAGE with MALDI-TOF/MS has found fourteen proteins with differential expression in comparison to uninfected brain tissue. Out of fourteen proteins, interestingly, thirteen proteins were down-regulated and only one protein has significantly higher expression (Farahtaj et al. 2013). As reported earlier, cytoskeleton-related proteins dynein/dynactin have a role in rabies virus transport along microtubules of neuronal cells (Raux

et al. 2000). Therefore, down-regulation of dynein light chain, centractin beta isoform and tubulin alpha-1C chain (TUBA1C) in rabid human brain tissue plays role in axonal trafficking and also lead to microtubular instability of neurons (Farahtaj et al. 2013).

Rabies virus infection in humans has also lead to affect proteins associated with metabolism such as fatty acid-binding protein 3 (FABP3), macrophage migration inhibitory factor (MIF), glutamine synthetase (GS) and alpha-enolase (ENO1). Energy production, signalling associated with long-chain polyunsaturated fatty acid in the brain as well as arachidonic acid uptake, trafficking and steady-state maintenance of brain lipid levels are various functions of FABP3 (Murphy et al. 2005). MIF plays role in the regulation of inflammatory stress response (Bacher et al. 1998). Protein oxidation in brain is susceptible to oxidative stress and is mediated by GS (Poon et al. 2006). Reduction in metabolism-associated proteins in rabies virus-infected brain tissue of humans possibly affects metabolism in neurons which is necessary for efficient neuronal function. ENO1 was only protein observed with higher-level expression in rabies virus-infected human brain tissue in support with earlier studies in mouse neuronal cells (Wang et al. 2011). As enolase 1 is a major glycolytic enzyme, higher expression of enolase 1 may affect other enzymes of glycolytic activity with an impact on brain metabolism (Farahtaj et al. 2013; Tholey et al. 1982).

A proteomics approach by iTRAQ with LC-MS/MS has identified four-hundred-two proteins in paralytic and furious human rabid patients when compared with uninfected patients. Several proteins of oxidative phosphorylation such as ATP5L, ATP5J2, ATP6V1E1, NADH: ubiquinone oxidoreductase core subunit S3, NADH: ubiquinone oxidoreductase subunit B4, NADH: ubiquinone oxidoreductase subunit AB, and ubiquinol-cytochrome c reductase, complex III subunit X were up-regulated in rabies virus-infected human brains (Venugopal et al. 2013). Energy generated from oxidative phosphorylation has been useful for rabies virus replication (Conti et al. 1990) as well as retrograde axonal transport with the involvement of several mitochondria (Goldstein and Yang 2000). Therefore, up-regulation of such proteins due to rabies virus infection may lead to mitochondrial dysfunction and glycolysis (Venugopal et al. 2013).

Calcium-calmodulin protein kinase II (CAMK2) is abundantly present in brain (Lisman et al. 2002) and expresses in astrocytes to protect against apoptosis (Song et al. 2006). Proteomics analysis in paralytic rabid cases has found up-regulation of calcium-calmodulin protein kinase II A (CAMKIIA) suggesting the importance of such kinase in neuronal signaling (Venugopal et al. 2013). According to earlier reports, glutamate ammonia ligase (GLUL) plays role in host defense mechanisms during simian immunodeficiency virus infection (Chrétien et al. 2002). Up-regulation of GLUL in rabid human patients suggests a protective mechanism against infection with rabies virus (Venugopal et al. 2013). In addition, programmed cell death 6 interacting protein (PDC6IP) causes apoptosis of neurons either by being dependent upon caspase or independent of caspase. Endosomal trafficking is another function associated with PDC6IP (Trioulier et al. 2004). Down-regulation of PDC6IP in

human brain due to rabies virus infection leads to neuronal dysfunction (Venugopal et al. 2013).

A recent study by iTRAQ with nanoLC-MALDI MS/MS proteomics approach has shown differential expression of nineteen proteins due to rabies virus infection in dog brain tissue in comparison to uninfected tissue. Out of nineteen proteins, ten proteins were down-regulated and nine proteins showed up-regulation (Behera et al. 2020). DEAD-box helicase 3 X-linked (DDX3X) protein has been observed involvement in viral replication during several virus infections such as hepatitis C virus, human immunodeficiency virus type 1, influenza virus, dengue virus, and japanese encephalitis virus (Bortz and García-Sastre 2011; Ishaq et al. 2008; Li et al. 2014, 2015; Randall et al. 2007). Therefore, down-regulation of (DDX3X) protein due to rabies virus may provide an antiviral response or may play role in viral replication as observed for several other RNA viruses (Behera et al. 2020).

In rabies virus-infected dog brain tissue samples, DLA-64 protein showed the highest expression level among all nineteen proteins. DLA-64 plays role in immune response by peptide presentation at both CD4+ and CD8+ T cells surface (Vyas et al. 2008). Previous findings have shown overexpression of rabies virus glycoprotein causes enhanced expression of major histocompatibility class I molecules (Yang et al. 2015). Higher expression of DLA-64 due to rabies virus infection suggests a role of host immune response against viral infection. (Behera et al. 2020).

3.4.4.2 Ebola Virus

Ebola virus (EBOV), is a *Filoviridae* family member with a single negative-strand RNA genome (Kourtis et al. 2015). In West Africa, an outbreak of ebola virus infection had maximum effect than several earlier ebola outbreaks (Frieden et al. 2014). Disease caused by ebola virus is highly virulent in both primates and humans. Analysis of changes in proteome profile during infection can generate pathogen-specific biomarkers. However, sometimes, complications arise in patients due to supportive care treatments (Dunachie et al. 2017; McElroy et al. 2014, 2016).

Proteomics analysis of purified virions from 1D-PAGE with LC-MS/MS has detected heat shock protein A5 in ebola virus (Spurgers et al. 2010). Heat shock protein A5 is a heat shock protein 70 family member and plays role in virus biology. Heat shock protein A5 has also reported as a chaperone in hepatitis B virus, sinbis virus (Awe et al. 2008; Mulvey and Brown 1995). Knockdown of heat shock protein A5 transcript has reduced infection with ebola virus (Spurgers et al. 2010). Therefore, heat shock protein A5 shows an impactful effect in ebola virus biology.

The use of non-human primates as model systems is beneficial for proteomic characterization in pathogen infection. During ebola virus infection in rhesus macaques, characterization of plasma proteome was carried out by LC-MS/MS along with a peptide tagging approach. Majority of host proteins showed alterations in expression level after day 4 or day 5 of ebola virus infection when compared with uninfected rhesus macaques (Ward et al. 2019).

Earlier transcriptomics study during ebola virus infection in non-human primates has shown cytokine expression is concurrent with onset of fever (Speranza et al. 2018). According to proteomics analysis, onset of fever due to ebola virus infection in rhesus macaques is related to alteration in protein expression. In addition, detection of viremia and development of pyrexia was also associated with proteome profile changes during ebola virus infection. But in some non-human primates, protein alteration was observed before detectable viremia and pyrexia (Ward et al. 2019).

Acute-phase proteins such as serum amyloid A2 (SAA2) and C-reactive protein (CRP) protein undergo elevated expression during bacterial infections in comparison to viral infections (dos Anjos and Grotto 2010; Holub et al. 2013; Lannergård et al. 2003; Yeung et al. 2004). Contrastingly, after 5 or 6 days of ebola virus infection, expression of CRP and SAA2 was similar with *Burkholderia pseudomallei* bacterium infection in non-human primates (Ward et al. 2019). Usually, the expression of acute-phase proteins increases with increase in severity of disease (Chen et al. 2015) but such distinguishable expression during ebola virus infection has put ebola virus disease in a separate class of viral infections.

Proteomics analysis has also found another acute-phase protein namely leucine-rich alpha-2-glycoprotein 1 (LRG1) with higher expression during ebola virus disease in rhesus macaques (Ward et al. 2019). During infection, LRG1 is produced by hepatocytes via interleukin-6 activation. Moreover, in disease conditions, neutrophils secrete LRG1 after activation (Shirai et al. 2009). Enhancement in LRG1 expression during ebola virus infection emphasizes both severity and degree of inflammation (Ward et al. 2019).

Ras-guanosine triphosphatase accelerating protein Src homology 3 (Ras-GAP SH3) domain-binding protein plays role in host-defense mechanism against virus infections. Ras-GAP SH3 domain-binding protein also acts as a surrogate biomarker for type-1 interferon-dependent gene activation (Grassadonia et al. 2004; Nielsen et al. 2014). Up-regulation of Ras-GAP SH3 domain-binding protein has been observed in hantavirus infection, human immunodeficiency virus infection and dengue virus infection (Hepojoki et al. 2014; Liu et al. 2016; Yang et al. 2014). Human patients of hantavirus and dengue virus infection have reported higher levels of Ras-GAP SH3 domain in their plasma (Hepojoki et al. 2014; Liu et al. 2016). Therefore, up-regulation of Ras-GAP SH3 domain during ebola virus infection may promote host defense mechanism.

Ribosomal proteins have protein chaperone activity and play role in the regulation of transcription process (Lindström 2009; Wool 1996). Proteomics analysis of ebola virus has identified four ribosomal proteins such as ribosomal protein L18 (RPL18), ribosomal protein L5 (RPL5), ribosomal protein L3 (RPL3), and ribosomal protein S6 (RPS6). Ribosomal proteins have a role in ebola virus biology because as per earlier studies, reduction in each ribosomal protein by small interference RNA technology leads to inhibition of viral replication (Spurgers et al. 2010). Interaction between RPL18 and P6 protein of cauliflower mosaic virus has been observed (Leh et al. 2000). RPL5 plays role in human immunodeficiency virus type 1 replication by interacting with the viral cofactor eukaryotic initiation factor 5A (Schatz et al. 1998). Similarly, RPL3 regulates ribosomal frameshifting in yeast M1

killer virus (Peltz et al. 1999). RPS6 regulates gene expression in several alpha herpes viruses. Association of RPS6 with non-structural protein 2 of alphavirus mediates expression from alphavirus messages (Montgomery et al. 2006). Due to involvement of ribosomal proteins in several virus biology, all four ribosomal proteins can provide insight into ebola virus biology.

3.4.4.3 Marburg Virus

Proteomics analysis of purified marburg virions from 1D-PAGE with LC-MS/MS has identified several host proteins. As per proteomics studies with ebola virus, heat shock protein A5 was not found in association with marburg virus. But, knockdown of heat shock protein A5 by small interference RNA technology has reduced rate of marburng virus infection. Moreover, release of marburg virus from infected cells was also inhibited due to reduction in heat shock protein A5 (Spurgers et al. 2010). Non-detection of heat shock protein A5 by proteomics approach was successfully found in western blot analysis in purified marburg virus (Kolesnikova et al. 2002). Role of heat shock protein A5 has been reported in human cytomegalovirus production and dengue virus serotype 2 production (Buchkovich et al. 2008; Limjindaporn et al. 2009). Inhibition of both human cytomegalovirus and dengue virus serotype 2 productions occurred due to reduction of heat shock protein A5.

In contrast to ebola virus, no ribosomal proteins were detected in marburg virus by proteomics analysis. Surprisingly, knockdown of ribosomal protein L18 and ribosomal protein L3 showed inhibition of marburg virus infection. But, small-interference RNA technology against ribosomal protein S6 and ribosomal protein L5 enhanced marburg virus infection moderately (Spurgers et al. 2010). Finding a link between ribosomal proteins and marburg virus is still plausible.

Marburg virus matrix protein VP40 directs budding of VP40-containing virus-like particles (VLPs) budding. Several host proteins like actin and tumor susceptibility gene 101 were found in ebola VP40-induced virus-like particles (Han and Harty 2005; Licata et al. 2003). However, actin and tumor susceptibility gene 101 were not detected in marburg VP40-dependent virus-like particles (Kolesnikova et al. 2009). Contrastingly, purified marburg virions showed expression of similar proteins by western blot analysis (Kolesnikova et al. 2002). Thus, contrasting behaviour in actin and tumor susceptibility gene 101 expression in marburg virus still needs investigations.

3.4.4.4 Severe Acute Respiratory Syndrome Coronavirus 2

At end of 2019, an acute respiratory distress syndrome started to spread throughout the world. The causative agent of acute respiratory distress syndrome was a novel corona virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu et al. 2020). Recently identified novel coronavirus belongs to *Coronaviridae* family with a single-stranded RNA as the genetic material (Wu et al. 2020a). Severe acute

respiratory syndrome coronavirus 2 infection is a zoonotic disease with primary transmission occurs through direct contact with either infected person or carriers (Li et al. 2020). Severe acute respiratory syndrome coronavirus 2 infected patients show symptoms like anosmia, fever, cough and dyspnea (Parma et al. 2020; Wu et al. 2020b). However, an infection may lead to severe respiratory illness and death in critical patients.

Multiplexed enhanced protein dynamics (mePROD) proteomics is a recently described proteomics technique to determine changes in proteome and translatome changes at high temporal resolution (Klann et al. 2020). mePROD proteomics with severe acute respiratory syndrome coronavirus 2 infected cancer-coli 2 (Caco-2) cells has quantified translation of two thousand seven hundred fifteen proteins (Bojkova et al. 2020).

Effects of several RNA viruses on translation have been well documented (Narayanan et al. 2008). Limited attenuation of translation and modulation of core cellular pathways was revealed by temporal proteome and translatome proteomics. In support of earlier reports, severe acute respiratory syndrome coronavirus 2 infection has shown mild reduction in expression of angiotensin-converting enzyme 2 (Bojkova et al. 2020; Jia et al. 2009).

In severe acute respiratory syndrome coronavirus and middle east respiratory syndrome coronavirus, host translation is targeted to inhibit viral replication (Pillaiyar et al. 2020; Shen et al. 2019). To determine changes in host cell translation process post severe acute respiratory syndrome coronavirus 2 infections, translatome and proteome study was carried out by use of two translation inhibitors namely cycloheximide and emetine. While cycloheximide inhibits translation elongation process, emetine inhibits 40S ribosomal protein S14. As a result, viral replication was prevented remarkably in infected cells. Therefore, infection with severe acute respiratory syndrome coronavirus 2 reshapes host translation process via increased production of host translation machinery components. An increase in host translation machinery components compensates for translation inhibition in an antagonistic manner for inhibitors (Bojkova et al. 2020). In the future, studies with other translation inhibitors can provide information for prevention of severe acute respiratory syndrome coronavirus 2 infections.

Proteomics analysis further identified higher expression of proteostasis, spliceosome and nucleotide biosynthesis pathways components. Any possible compound of enriched cellular pathways can enable the determination of potential drug targets against severe acute respiratory syndrome coronavirus 2 infections (Bojkova et al. 2020).

Protein complexes are studied by combining cross-linking and LC-MS/MS approaches (Haupt et al. 2017). During viral lifecycle, protein-protein interactions play several vital functions (Varjosalo et al. 2013). Affinity purification liquid chromatography-tandem mass spectrometry (AP-LC-MS/MS) analysis, has shown higher expression of proteins of neutrophil activation, blood coagulation and lower expression of T cell receptor signaling protein in PBMCs of severe acute respiratory syndrome coronavirus 2 patients in comparison to healthy patients (Li et al. 2020).

Proteomics and interactome studies with severe acute respiratory syndrome coronavirus 2 infected patients have shown interactions between human inflammatory and immune response key players and several viral non-structural proteins (Li et al. 2020). Nuclear factor-kappa-B repressing factor plays role in neutrophil chemotaxis via induction of interleukin-8 (Huang et al. 2014a; Nourbakhsh et al. 2001). Viral non-structural protein 10 of severe acute respiratory syndrome coronavirus 2 may play some role in the regulation of interleukin-8 levels by targeting nuclear factor-kappa-B repressing factor. Therefore, the interaction between viral non-structural protein nsp10 and nuclear factor-kappa-B repressing factor (NKRF) may shape host immune signature during severe acute respiratory syndrome coronavirus 2 infection (Li et al. 2020).

Upon viral infection, TANK-binding kinase 1 phosphorylates key transcriptional factors, interferon regulatory factor 3 and interferon regulatory factor 7 (Fitzgerald et al. 2003). TRAF family member-associated nuclear factor-kappa-B (TANK)-binding kinase 1 also causes nuclear factor kappa B activation via a ternary complex formation with TANK and tumor necrosis factor receptor-associated factor 2 to facilitate nuclear factor-kappa-B activation (Pomerantz and Baltimore 1999). Interaction among open reading frame 6 and ternary complex may modulate type I interferon and nuclear factor-kappa-B pathways. Thus, such interaction allows viral escape from host immune response. The activation of several signal transducers and activators of transcription are activated by a tyrosine-protein kinase KIT (Chaix et al. 2011). Upon interferon or interleukin-6 stimulation, signal transducers and activators of transcription are involved in signal transduction. During severe acute respiratory syndrome coronavirus 2 infection, association between tyrosine-protein kinase KIT and open reading frame may lead to the regulation of interferon and interleukin-6 signaling pathways (Li et al. 2020).

An integrative proteo-transcriptomics analysis with TMT pro labeling and LC-MS/MS in severe acute respiratory syndrome coronavirus 2 infected hepatocyte-derived cellular carcinoma (Huh7) cells have found a modulation of mammalian target of rapamycin (mTOR) signaling, hypoxia-inducible factor 1 (HIF-1) signaling, tumor necrosis factor (TNF) signaling (Appelberg et al. 2020). According to earlier studies, mTOR signaling regulates apoptosis, cell survival, host transcription, translation processes and several RNA viruses have hijacked mTOR signaling (Ehrhardt and Ludwig 2009; Kindrachuk et al. 2015; Mizutani et al. 2005; Ranadheera et al. 2018). Activation of eukaryotic translation initiation factor 4E and eukaryotic translation initiation factor 4E-binding protein 1 is carried out by mTOR signaling complex 1 and a serine/threonine-specific protein kinase B. Such activation of eukaryotic translation initiation factors leads to translation of HIF-1 alpha for transcription and translation of specific host genes. Moreover, tumor necrosis factor (TNF) signaling is linked to hypoxia-inducible factor 1 signaling (Zhou et al. 2004). Tumor necrosis factor 1 signaling leads to dysregulation of caspase 8, caspase 10, major proteins of interferon signaling as well as nuclear factor-kappa-B signaling pathways (Chen et al. 2017). In agreement with earlier reports regarding role of interferon-beta in coronavirus infection, alterations in signaling pathways can be targeted against viral infection (Appelberg et al. 2020).

Shotgun proteomic workflow such as nano-HPLC coupled with Orbitrap-MS/MS approach has identified unique peptides from viral nucleoprotein in gargle samples of severe acute respiratory syndrome coronavirus 2 patients. Identification of unique peptides from virus can be helpful in development of diagnostic tools for severe acute respiratory syndrome coronavirus 2 (Ihling et al. 2020). From nasopharyngeal epithelial swab samples of severe acute respiratory syndrome coronavirus 2 infected patients, peptides of viral nucleocapsid protein were also identified by LC-MS/MS proteomics approach (Nikolaev et al. 2020). Contrastingly, LC-MS/MS based proteomics approach on patients' nasopharyngeal epithelial swab samples provided confident identification of viral nucleoprotein even at lower viral load. Parallel mass spectrometric-based approaches can be developed for viral detection in several other samples like nasopharyngeal mucosa, sputum, saliva and other biological fluids. (Nikolaev et al. 2020).

Moreover, a combination of targeted proteomics and metabolomics assays in serum samples of severe acute respiratory syndrome coronavirus 2 infected patients have found dysregulation in lipid metabolism due to down-regulation of multiple apolipoproteins such as apolipoprotein A-1, apolipoprotein A-2, apolipoprotein H, apolipoprotein L1, apolipoprotein D and apolipoprotein M in serum of severe acute respiratory syndrome coronavirus 2 infected patients. Majority of identified apolipoproteins remain associated with macrophage functions. Therefore, down-regulation of apolipoproteins in serum of severe acute respiratory syndrome coronavirus 2 patients may affect macrophage functions in comparison to uninfected patients (Shen et al. 2020). Steroid hormones such as progesterone, androgens and estrogens promote macrophage activity (Cain and Cidlowski 2017). Increased expression of 21-hydroxypregnenolone, an intermediate of corticosterone biosynthesis deciphers a protective mechanism against severe acute respiratory syndrome coronavirus 2 infection (Shen et al. 2020).

Following severe acute respiratory syndrome coronavirus 2 infection, one hundred five proteins undergo differential expression, out of which ninety-three proteins had specific modulation. Furthermore, fifty proteins were found to be involved in three major pathways such as macrophage function, activation of complement system and platelet degranulation. Among metabolites, three hundred seventy-three metabolites showed significant alterations with the involvement of two hundred four metabolites in disease severity. Significantly altered eighty metabolites also involved in macrophage function, complement system activation and platelet degranulation pathways. Therefore, in severe acute respiratory syndrome coronavirus 2 patients, deregulation of proteins and metabolites showed metabolic and immune dysregulation (Shen et al. 2020).

A proteomics approach by nanoLC-MS/MS analysis in nasopharyngeal samples from severe acute respiratory syndrome coronavirus 2 patients have shown alterations in neutrophil and platelet degranulation and immune system response in comparison to uninfected patient samples (Akgun et al. 2020). Thus, innate immune response and cytokine production are considered as severe acute respiratory syndrome coronavirus 2 mediated host responses.

3.5 Conclusion

Viruses undergo adaptation inside a host organism and carry out modifications into host's cellular machinery. Upon viral infection, several host proteins, as well as viral proteins, alter their expression levels. Proteomics is an advanced high throughput tool through which identification as well as quantitation of different proteins can be achieved to decipher the involvement of the altered proteins during viral infections.

This review has described several proteomics technologies and approaches along with their advancement over time.

Moreover, we have also reviewed the viral proteomics studies on most common animal viruses broadly categorized as animal viruses, human viruses and zoonotic viruses to understand the role of proteins altered during viral infection. Proteomics approaches have successfully found major altered proteins due to several virus infections and understanding their probable role in viral pathogenesis.

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Chapter 4
Influence of the Ovarian Reserve and Oocyte Quality on Livestock Fertility

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Abstract Functional traits of livestock species such as health and reproduction have a substantial effect on profitability. Poor reproduction limits livestock productivity, resulting in economic loss. Fertility in livestock species is leaned over the years as the demand for livestock products is increasing. The most common causes of decline in fertility are diseases, nutrition, genetics, management, gamete quality, and quantity. Primarily, the fertility lifespan of the mammalian female depends on the pool of ovarian reserves and their quality. Animals with declined or fewer ovarian reserves are known to be inferior in fertility. A considerable loss of ovarian reserve occurs during oogenesis, where more than 99% of the oocytes undergo apoptosis. The remaining oocytes may fail during the maturation process with poor oocyte quality. Although, the emerging field of assisted reproductive technology (ART) has provided novel avenues for a manifold increase in livestock production. Still, the quantity and the quality of the oocytes carried by the animal play a significant role in the fertility outcome. Despite the massive clinical significance of the maintenance of ovarian reserves and oocyte quality, the molecular mechanisms and their impact on livestock fertility are scanty.

This chapter reviews the mechanisms of maintenance and the factors that affect the ovarian reserve and oocyte quality in livestock species. We discuss the influence of ovarian reserves on female fertility and the causes of oocyte loss at early and late stages. Also, we illustrate quality control checkpoint pathways to maintain the

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oocyte reserves. Furthermore, we describe the effect of ovarian disorders on livestock follicle reserve, growth, and health. Finally, we analyze the emerging biotechnological interventions to improve oocyte reserve and quality.

Keywords Assisted reproductive technology (ART) · Livestock · Fertility · Oocyte · Ovarian reserve

Abbreviations

3MA	3-Methyladenine
3MC	3-Methylcholanthrene
4E-BPs	Eukaryotic translation initiation factor 4E-binding protein 1
AFC	Antral follicle count
AMH	Anti-mullerian hormone
AMPK	AMP-activated protein kinase
ART	Assisted reproductive technology
ATG14	Autophagy related 14
ATG7	Autophagy related 7
ATM/ATR	Ataxia telangiectasia mutated/Ataxia telangiectasia and Rad3-related protein
AZT	Azidothymidine
BAK	BCL2 Antagonist/Killer 1
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
BCL2L1	BCL2 Like 1
Beclin1	Coiled-coil, moesin-like BCL2 interacting protein
BH3	Bcl-2 Homology 3
BLIMP1	B lymphocyte-induced maturation protein-1
BMP	Bone morphogenetic proteins
CO	Crossover
cAMP	Cyclic adenosine monophosphate
Caspase 2	Cysteine-aspartyl protease-2
caspase 9	Cysteine-aspartyl protease-9
caspase-3	Cysteine-aspartyl protease-3
cCASP3	Cleaved Caspase-3
cGMP	Cyclic guanosine monophosphate
CHEK 2	Checkpoint kinase 2
cIAP1	Cellular inhibitor of apoptosis 1
cIAP2	Cellular inhibitor of apoptosis 2
CK1	Casein kinase 1
CL	Corpus luteum
CNP	C-type natriuretic peptide
COC	Cumulus-oocyte complex

DDR	DNA damage response
DHEA	Dehydroepiandrosterone
Dmc1	DNA Meiotic Recombinase 1
DNA	Deoxyribonucleic acid
DPC	Day Post Coitus
DPP	Day post-partum
DSB	Double-strand break
EGF	Epidermal growth factor
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
EOA	Early oocyte attrition
ESCs	Embryonic stem cells
FASL	Fas ligand
FF	Follicular fluid
FOA	Fetal oocyte atresia
FOXO 3A	Forkhead box O3
FSH	Follicle-stimulating hormone
GC	Granulosa cell
GnRH	Gonadotropin-releasing hormone
GSK3	Glycogen Synthase Kinase-3
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
GVT	Germinal vesicle transfer
hCG	Human chorionic gonadotropin
HMGB1	High Mobility Group Box 1
ICSI	Intracytoplasmic sperm injection
IIS	Insulin/insulin-like growth factor signalling
IL6	Interleukin 6
ILK	Integrin Linked Kinase
iPSc	Induced pluripotent stem cells
IVF	In-vitro fertilization
IVM	In-vitro maturation
LC3	Light Chain 3
LC3B	Light Chain 3B
LH	Luteinizing hormone
LhX8	LIM homeobox 8
LINE-1(L1)	Long interspersed element-1
LKB1	Liver kinase B1
LOA	Late oocyte attrition (LOA)
Mael	Maelstrom spermatogenic transposon silencer
MI	Metaphase I
MII	Metaphase II
MLH1	MutL homolog 1
MLKL	mixed-lineage kinase domain- like protein
MRI	Magnetic resonance imaging

mRNA	Messenger RNA
MRT	Mitochondrial replacement therapy
MSH4	MutS Homolog 4
mTOR	Mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
MVH	Mouse VASA homolog
NAD	Nicotinamide adenine dinucleotide
NEB	Negative energy balance
NLRs	NOD-like receptors
NMN	Nicotinamide mononucleotide
Nobox	Newborn ovary homeobox protein
NOD	Nucleotide oligomerization domain
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NPR2	Natriuretic peptide receptor 2
NSN	Non-surrounded nucleolus
OT	Ooplasmic transfer
p53	Tumor protein p53
PARP1	Poly-ADP ribose polymerase-1
PB1T	Polar body 1 transfer
PCD	Programmed cell death
PCOS	Polycystic ovary syndrome
PDE3A	Phosphodiesterase 3A
PDK1	Pyruvate Dehydrogenase Kinase 1
PET-CT	Positron emission tomography- Computed Tomography
PGCLCs	Primordial germ cell-like cells
PGCs	Primordial germ cells
PGF2α	Prostaglandin F2α
PI3K	Phosphoinositide 3-kinase
PI3K/Akt	phosphatidylinositol 3-kinase/Protein kinase B
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
piRNA	Piwi-interacting RNA
PNT	Pronuclear transfer
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
RIP1	Receptor-Interacting Protein kinase 1
RIPK3	Receptor Interacting Serine/Threonine Kinase 3
RNF212	Ring Finger Protein 212
ROS	Reactive oxygen species
rpS6	Ribosomal protein S6
RTK	Receptor tyrosine kinases
S6K1	Ribosomal protein S6 kinase beta-1
SHBG	Sex hormone binding globulin
SN	Surrounded nucleolus

SOX17	SRY-related HMG-box 17
ST	Spindle chromosome transfer
Stra8	Stimulated by Retinoic Acid 8
SUMO	Small Ubiquitin Like Modifier
Sycp1	Synaptonemal Complex Protein 1
SYCP3	Synaptonemal Complex Protein 3
TAI	Timed artificial insemination
Tap63	Trans-activating isoform of p63
TAp63α	Trans-activating isoform of p63
TGF-beta	Transforming growth factor beta
TLRs	Toll-like receptors
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor alpha
Tsc1	Tuberous sclerosis proteins 1
Tsc2	Tuberous sclerosis proteins 2
VDAC2	Voltage Dependent Anion Channel 2
WNT	Wingless-related integration site
ZP	Zona pellucida
ZVAD	Z-VAD- FMK
γ- H2AX	gamma – H2A histone family member X

4.1 Introduction

The livestock sector contributes a majority share to the ever-increasing demand for quality food by increasing human population, urbanization, and social mobility. According to the World Migration Report (2015), the urban population is growing up to approximately 6.4 billion by 2050, which is roughly 1.5 times more than the present population. Steadily increasing world population results in both challenges and opportunities, especially in low- and middle-income countries result in increases in the demand for milk, meat, and other animal based-products in rapidly developing and populated countries (Martin et al. 2004; Wu and Zhu 2014; Kumar et al. 2019a, b). Animal health and reproduction are the two critical factors that contribute to fulfilling the growing demands of animal-based products. Farmers are looking for superior animals with high prolificacy and fecundity with minimum cost. However, poor fertility in livestock limits productivity results in millions of dollars of loss annually across the globe (Williams 2013).

Causes of infertility are numerous, such as infections, malnutrition, genetic disorders, chromosome abnormalities, and testis or ovarian disorders (Ibraheem and Ramachandran 2003; Khan et al. 2016). Especially in females, reproductive problems cause infertility, and sub-fertility incurs considerable losses to the livestock sector. Unlike male animals, which continuously produce sperm throughout their life, females are born with a limited number of oocytes. Ovarian reserves or oocyte numbers dictate the fertility lifespan of the female. Any abnormalities in the

reproductive cycle or with the ovary affect the quantity and quality of the oocytes. For example, in beef cattle, the fertilization rate for oocytes is ~90%. In contrast, average calving rates to a single service are between 40% and 55%, suggesting a rate of embryonic/fetal mortality (excluding fertilization failure) of about 35–50%. The majority of embryonic loss (70–80%) occurs in the first 3 weeks of pregnancy. Poor quality of the gametes may lead to compromised quality of the embryo that drives to early loss of the embryo. Although, recent advancements in ART (Assisted reproductive technologies) improved the overall livestock reproductive potential. Still, the efficiency of the ART ultimately depends on the quantity and quality of the oocytes. Therefore, a thorough understanding of oocyte quality control mechanisms and novel biotechnology-based interventions are needed to maintain the quantity and quality of the eggs in livestock. Thus, in this chapter, we discuss the mechanisms of maintaining the oocyte reserve and their quality. Further, we reviewed the novel biotechnological or pharmacological treatment strategies that can be implemented in the livestock sector to enhance the quantity and quality of oocyte reserve.

4.2 Genesis and Development of Primary Oocytes

Germ cells originate as primordial germ cells (PGCs) outside the epiblast during embryogenesis (Fig. 4.1). In mammals, germ cell differentiation is first evident at the time of gastrulation with the help of intracellular signaling. In the pig, PGCs arise from the epiblast by succeeding up-regulation of BLIMP1 and SOX17 in response to WNT and BMP signaling (Kobayashi et al. 2017). Subsequently, nascent PGCs proliferate and migrate through the developing hindgut into the genital ridges (Fig. 4.1) (Buehr 1997). However, some vertebrates, such as birds, fish, and frogs, have acquired a different approach to germline segregation. It occurs much earlier in these species through the action of maternally supplied RNAs called germplasm (Strome and Updike 2015). PGCs are colonized in the genital ridges and continue to proliferate mitotically and develop into oogonia (Fig. 4.1). In mice beginning at ~13 dpc (Day Post Coitus), in response to retinoic acid produced from mesonephros. Further, Stra 8 (stimulated by retinoic acid gene 8) is transcriptionally up-regulated to initiates the DNA replication followed by activation of meiosis-specific genes (Baltus et al. 2006; Anderson et al. 2008). In humans, meiosis initiation partially depends on the retinoic acid (Le Bouffant et al. 2010). Whereas in chicken, germ cells enter into meiosis around E15.5 days in the left ovary, but the stra8 expression starts at E12.5 (Smith et al. 2008). Unlike mammals, in chickens, retinoic acid is produced in the embryonic gonads instead of the mesonephros (Yousefi Taemeh et al. 2019). The oogonia become primary oocytes as they arrive into meiotic prophase I (Fig. 4.1). Then the oocytes continue in prophase I to finish events like replication followed by the formation of the synaptonemal complex and homologous recombination between homologous chromosomes synchronously in most of the species. Unlike males in females, all these events take place during gestation (Fig. 4.1). The timing of the meiotic prophase I events varies from species to species. However, in livestock species, these processes are conserved.

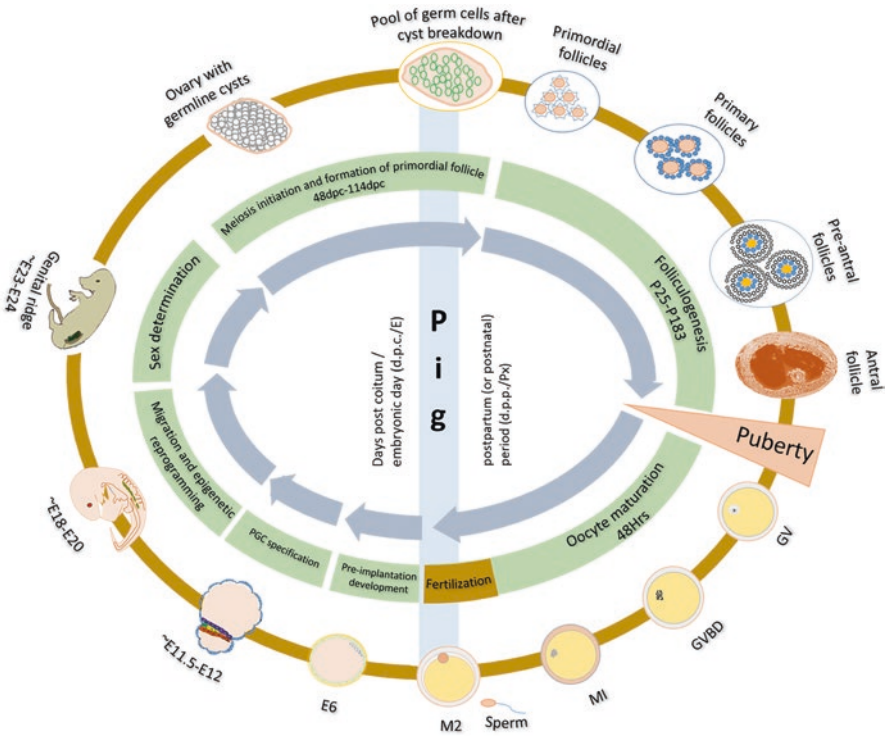

Fig. 4.1 Developmental cycle of Porcine female germline. Through a sequential division of fertilized oocytes develops into the blastocyst. Then the blastocyst is attached to the endometrial surface of the uterus and undergoes gastrulation to form all three germ layers. Porcine primordial germ cells (PGCs) are identified during gastrulation during ~ E12 (embryonic day 12). Then, the PGCs move near the yolk sac adjacent to the allantois. Further, PGCs relocate through the hindgut to the genital ridges, followed by germline cyst formation at ~E24. Then during the meiosis, oocytes undergo the quiescent stage after diplotene ~E48. After parturition, germline cysts break down to form primordial follicles. Later, the primordial follicles develop into primary follicles followed by preantral and antral follicles through the help of intra-and extra ovarian factors. Subsequently, under optimal hormonal signaling through cyclic recruitment, one or more antral follicles mature into preovulatory follicles. Finally, meiotic maturation starts with germinal vesicle breakdown (GVBD), followed by the first meiotic division where homologs are separated to form the first polar body, and followed by an arrest at metaphase II (MII stage) and wait for fertilization

4.3 Influence of Ovarian Reserves on Female Fertility

Ovarian reserves or oocyte pool indicate the resting egg quantity or supply that can produce offspring. These oocyte reserves deplete with the rise in the mother's age. In most of the species, oocyte numbers peak during mitosis to meiosis transition (Fig. 4.1). In humans, around 6–7 million oocytes enter meiosis (Table 4.1); by birth, only 1–2 million survive, and at the onset of puberty, the ovaries contain around 250,000 oocytes (Baker 1963). At the time of menopause, oocyte numbers

Table 4.1 Oocyte reserve dynamics across species

Species	Initial oocyte number	Oocyte number at birth	Oocyte number at puberty	Fertilizable oocytes	Oocyte loss (%)
Women	7,000,000	700,000	300,000	400	90
Cow	2,100,000	135,000	65,000	150	95
Rodents	75,000	10,000	500	70	80
Ovine or Ewes	900,000	82,000	55,000	200	91
Porcine or Sows	1,200,000	500,000	150,000	300	58
Bovine	2,700,000	135,000	–	–	95

drastically reduce to 100–200. In bovine, it gets reduced to approximately 1% of its original number, i.e., 2.7 million (Erickson 1966). Several groups (Ireland et al. 2010; Mossa et al. 2010) found a good correlation between ovarian reserves and fertility outcomes in female cattle. Particularly cattle with less antral follicle count (AFC) showed reduced conception rates and longer calving intervals.

Moreover, cattle with fewer antral follicles show impaired fertility and reduced reproductive hormones like an anti-Mullerian hormone (AMH) and progesterone in the circulation (Ireland et al. 2010; Mossa et al. 2010). Further, females with low AFC respond poorly to superovulation procedures and assisted reproductive techniques with low oocyte quality. Like bovine, Ewes also lose approximately 91% of the ovarian reserves around birth (Fig. 4.2) (Gustavo Adolfo Palma et al. 2012). Surprisingly, sows retain about 42% of the oocytes after early meiosis (Table 4.1) (Ireland et al. 2010; Mossa et al. 2010). Out of them, approximately 50,000 oocytes mature into fertilizable oocytes in a pig's lifetime. The reduced oocyte death in pigs could be the reason for higher fecundity and prolificacy. However, the molecular mechanisms for lower oocyte death are not known. The continuous decline of oocytes throughout a female lifetime is called atresia. Cumulatively, these pieces of evidence suggest that oocyte reserves are significant for female fertility. Our laboratory is interested in understanding the mechanisms of maintenance of the oocyte reserves in livestock species. We discussed some of our unpublished data in the below section.

4.4 Causes of Oocyte Atresia

Two types of atresia are classified based on the timing of the oocyte death. The early oocyte attrition (EOA) causes the loss of ~80% of all oocytes between 15.5 days post coitus (dpc) and 18.5 dpc in mouse models (Malki et al. 2014). Similarly, the late oocyte attrition (LOA) occurs in the postnatal period, where the germ cells in the quiescent phase or resting phase (Di Giacomo et al. 2005; Klinger et al. 2015; Qiao et al. 2018).

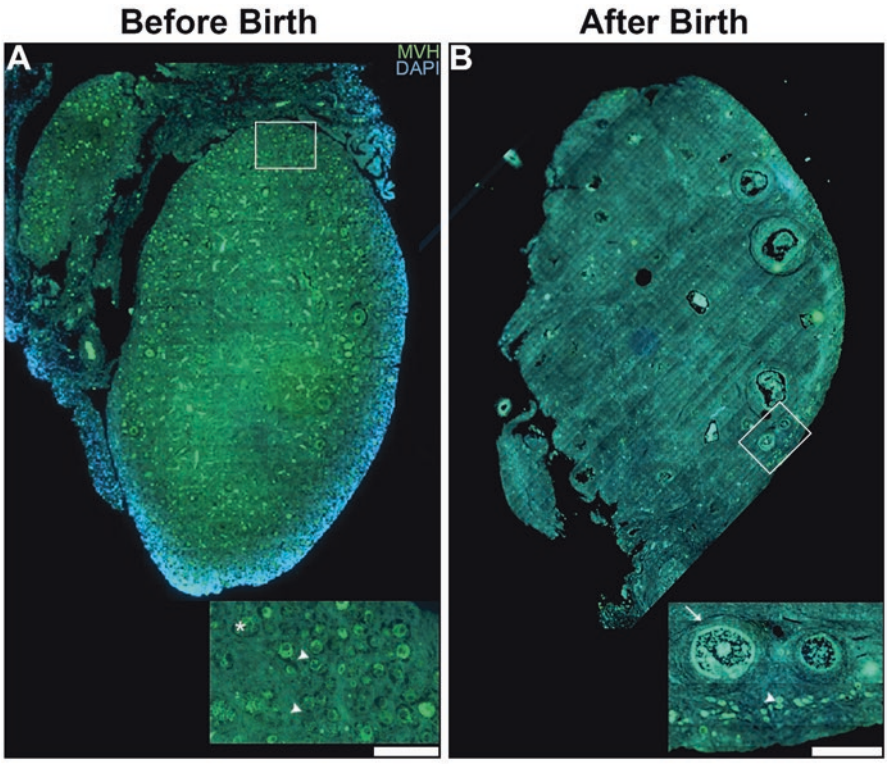

Fig. 4.2 Drastic reduction of follicles after birth in goat. (**a**, **b**) Before and after birth, goat ovaries were immunostained for MVH (green) and DNA (Blue). Cross-section images in the bottom panels are from the indicated region from the ovaries. Asterisk (*) indicates follicular cyst; arrowheads highlight immature primordial follicles; arrows indicate the growing follicles. Scale - 100 μm

4.4.1 Early Oocyte Atresia

Early oocyte atresia is the process where more than 80% of the oocytes are eliminated before birth (Kurilo 1981). This process is conserved among the vertebrates (Matova and Cooley 2001). In mice, fetal oocyte loss occurs throughout the meiotic prophase I. Observations in bovine suggest that early oocytes die those fail to enter into the primordial follicle stage (Ohno and Smith 1964). Our observations in the goat suggest a considerable loss of oocytes before birth (Fig. 4.2). The best-accepted theories for oocyte culling are death by neglect, death by defect, and death by sacrifice (Malki et al. 2014). All these theories involve some form of programmed cell death, either apoptotic or non-apoptotic. In mice, an abundant retrotransposon LINE-1 (L1) is associate with early oocytes atresia (EOA). The L1 expression is regulated epigenetically as DNA hypermethylation suppresses it. During the migration from the genital ridge, PGC undergoes epigenetic reprogramming. Demethylation of chromatin in PGC leads to the activation of the L1 element

(Fig. 4.1) (Goodier and Kazazian 2008; De Felici and Klinger 2011; Seisenberger et al. 2012; Deniz et al. 2019). This inference came from the work on fetal oocyte atresia (FOA), where a null mutation of the transposon silencer gene Mael caused a 2.4-fold rise in L1 expression that resulted in a three-fold drop in fetal oocytes at birth (Fig. 4.6). Further, retrotransposon inhibitor azidothymidine (AZT) suppresses the oocyte drop in Mael mutation by interrupting the L1 RNA-DNA hybrids (Malki et al. 2014). There are no bits of evidence found in other mammals for the presence of LINE-1 dependent fetal oocyte atresia. However, the presence of an active piRNA pathway during second trimester of human ovaries, primordial follicular stage in bovine, and pig raises the possibility of the existence of inhibitory mechanisms of LINE 1 in late mid prophase I of meiosis but not the early stages (Russell et al. 2017; Roovers et al. 2015). Alternatively, the overall less abundance of the piRNA in early stages may activate the LINE 1 transposons in higher-order vertebrates. Also, we cannot rule out the possibility of LINE 1 independent early oocyte attrition pathways.

4.4.2 Late Oocyte Atresia

Late oocyte atresia (LOA) can be separated into two phases. At the early stages after birth, the oocytes undergo apoptosis because of the errors in meiotic prophase I events. Sexually reproducing eukaryotes utilize a specialized cell division to produce haploid gametes called meiosis. Meiosis starts with one round of DNA duplication followed by two rounds of cell division. During meiotic prophase I, the linkage between homologous chromosomes needs to be established to assure proper segregation (Fig. 4.3). These inter-homolog linkages are generated by a precisely organized process called crossing over, followed by homologous chromosome

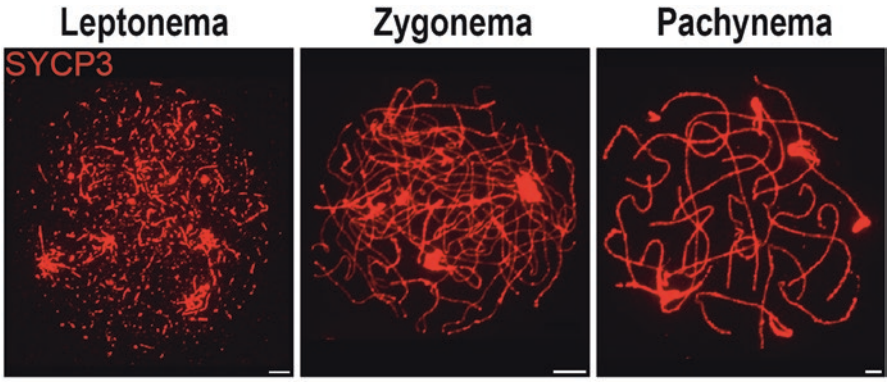

Fig. 4.3 Synoptonemal complex assembly in goat. Oocyte nuclei from goat fetal ovaries immunostained for SYCP3 (red) in all respective stages, i.e., Leptonema, Zygonema, and Pachynema. Scale bars = 10 μm

pairing and synapsis (the connection between homologs by a meiosis-specific zipper-like proteinaceous structure called the synaptonemal complex) (Hunter 2007, 2013). Meiotic recombination starts with double-stranded DNA break (DSB) formation by an evolutionarily conserved protein Spo11 (Fig. 4.6). After that, Spo11 is removed from the DNA, followed by 5′ strands resection to obtain 3′ single-stranded tails. Further, a sequence of reactions dependent on Rad51 and Dmc1, 3′ single-stranded tails undergo strand invasion to form recombination intermediates. Later MSH4 and RNF212 will be recruited and co-localized to stabilize the cross-over (CO) intermediates through complicated and strictly regulated processes to get the crossover outcome. The repair of established DSB can result in crossover through the exchange of homologous chromosome arms or a non-crossover without exchanging the homologous chromosome arms. At least in the mice, only a minor proportion (~10%) of DSBs mature into COs. However, each homolog pair obtains at least one CO.

The female germline is known to experience frequent gamete aneuploidy due to chromosome missegregation during meiosis. For example, the aneuploidy rate in female cattle 15 folds more than the male. Similarly, reports suggest that litter size is decreased in sows due to an increase in aneuploidy. Besides, younger pigs less than 1 year show a two-fold rise in aneuploidy than adults. Other livestock species like equine shows 5.5% aneuploidy (Szczerbal and Switonski 2016). One of the major reasons for 75% of the early-stage human oocyte loss is abnormalities in synapsis and recombination. Also, meiotic pairing defects are common in spermatocytes than oocytes (Speed 1988). Although meiotic errors in livestock species fetal ovaries are not extensively studied, a study from bovine fetuses between 82 and 131 days using surface spread shows oocyte degeneration in all stages and localized to explicitly medullary cords. The oocyte degeneration increases with the age of the fetus. Surprisingly, synapsis and pairing defects rose from 35.2% during 80–80 days to 69.3% in 111–130 days fetuses. These kinds of meiotic synaptic defects are less in healthy bulls, approximately 9% compared to 69% in cows (Koykul et al. 2000). Our laboratory found more synaptic and meiotic recombination defects in goat oocytes than spermatocytes (Figs. 4.4 and 4.5). Taken together, these pieces of evidence suggest that the significant loss of oocytes in the initial stages occurs because of the synapsis and meiotic recombination defects (Figs. 4.4 and 4.5). Based on abnormalities, oocyte apoptosis can be classified into DNA damage dependent and DNA damage independent. In mice, the absence of synaptonemal complex proteins Sycp1 and Sycp3 induces the oocytes to die between 4 and 8 days after birth. However, without a synaptonemal complex where Sycp1 and Sycp3 double mutant oocytes survive longer than single mutants. Few oocytes survive up to 60 days post-partum (dpp) in Spo11 null, where the oocytes fail to form canonical DSBs results in synapsis defects (Kouznetsova et al. 2011) (Fig. 4.6). On the other hand, depletion of Dmc 1, a Rec A homolog required for interhomolog repair of meiotic DSBs, shows a more severe effect than spo11 null. Similar to Sycp1 mutant in Msh4 null where the recombination is defective, ovaries completely get degenerated by 4dpp. However, the oocyte pool increases with the depletion of Rnf212, a SUMO E3

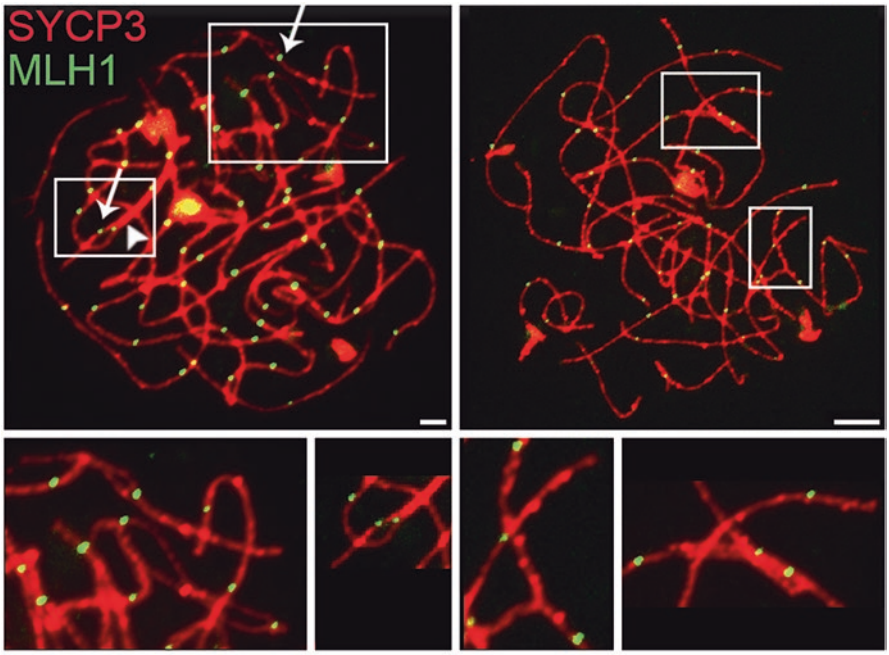

Fig. 4.4 Synapsis defects in goat oocytes. Goat fetal oocyte nuclei were immunostained for axis marker SYCP3 (Red) and the crossover marker MLH1 (Green). White square highlights the pseudo synapsis. Scale bars = 10 μm

ligase required for crossover formation. Although all of the oocytes collapse at MI, surprisingly, the Rnf212 mutant rescues the Msh4 single mutant oocyte death by promoting the inter-sister DNA recombination (Qiao et al. 2018). At least from the available mouse data, we can emphasize the importance of early meiotic prophase events and their roles in oocyte apoptosis. Further, the existing data suggest the interplay between the synaptonemal complex and recombination is necessary to maintain the oocyte reserves and quality. During the late phase of folliculogenesis based on the histological evidence, two forms of atresia were witnessed in bovine: 1. Antral atresia, which is more prominent and occurs throughout the follicle development stages, where the granulosa cells undergo apoptosis near the antrum. The characteristics of this form of atresia include pyknotic nuclei, fragments of mitochondria, and plasma membrane in the granulosa layers near the antrum. 2. Basal atresia, which is unique to small follicles less than 5 mm in diameter characterized by the carnage of the basal membrane, which supports the theca cells, invading capillaries, and leukocytes. (Irving-Rodgers et al. 2001). In the pig, because of the granulosa cells apoptosis, the rate of atresia increases in the 3–5 mm diameter follicles. Further, transcriptome analysis revealed that 450 genes were differentially expressed between healthy and atretic follicles, where predominantly saw the crosstalk between apoptosis and autophagy genes (Fig. 4.6) (Zhang et al. 2018). Similarly, caspase-3 mediated follicular atresia observed in goats throughout the

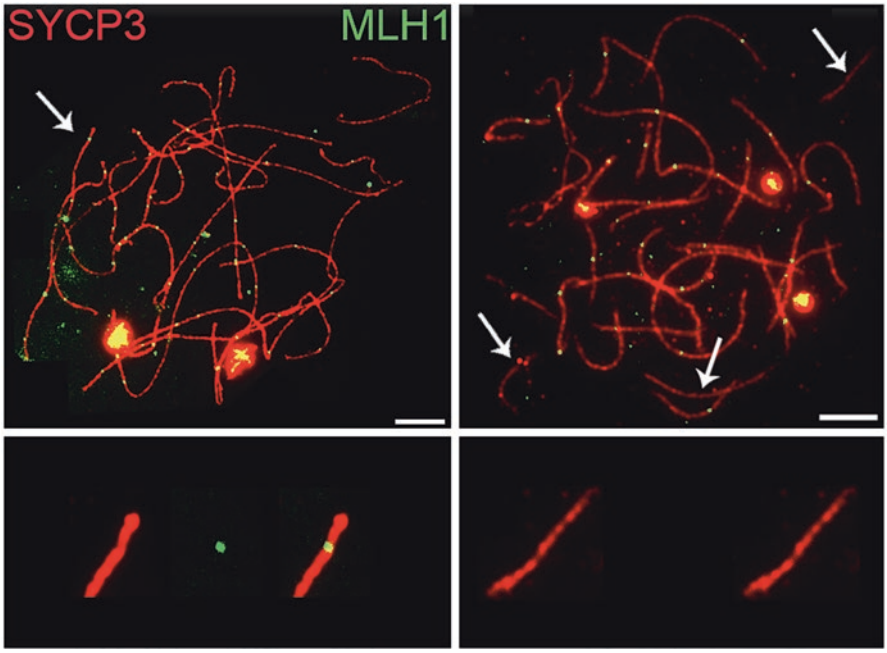

Fig. 4.5 Weak crossover obligation in goat oocytes. Goat fetal oocyte nuclei were immunostained for axis marker SYCP3 (Red) and the crossover marker MLH1 (Green). Arrow indicates the cropped homologs. The bottom left panel indicates homolog with crossover, and the right panel indicates the homolog without crossover. Scale bars = 10 μm

folliculogenesis (Kassouri-Maouche et al. 2018). However, most of these studies heavily depended on morphological characters, and few investigations concentrated on molecular and biochemical characterization of oocyte atresia in livestock species. Further, focused efforts are necessary to understand the complete molecular mechanisms in livestock species.

4.5 Quality Control Mechanisms in Oocyte Atresia

4.5.1 Mammalian Target of Rapamycin Dependent Oocyte Activation

During development, the oocyte has to undergo quality control checkpoints. mTOR, being one of these checkpoints, has a crucial role in the primordial follicle stimulation. Mammalian target of rapamycin (mTOR), a member of the PI3K-related kinase family, forms two distinct complexes; mTOR complex- 1 (mTORC1) and mTOR complex- 2 (mTORC2) (Sarbassov et al. 2005; Guertin and Sabatini 2007).

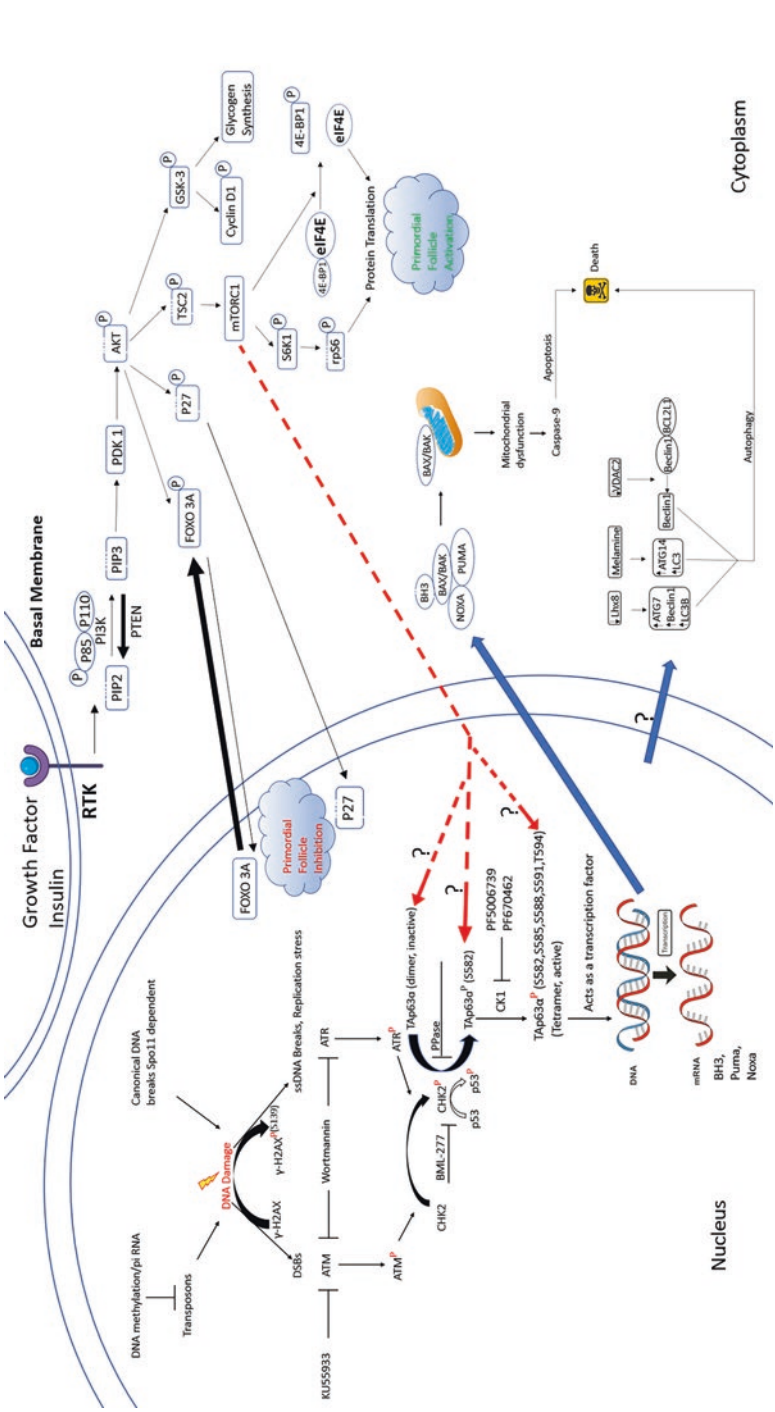

Fig. 4.6 Pathways responsible for the maintenance of ovarian reserve. In response to growth factors like insulin, PI3K/Akt signaling pathway activates the mTORC1 and FOXO 3A. mTORC1 initiates the activation of the primordial follicle by activation of rpS6 and eIF4E through phosphorylation of S6K1 and 4E-BPs, respectively. In response to the DNA damage, the CHEK2 and CK1 phosphorylate the TAp63α. Phosphorylation-induced tetramerization of TAp63α transcriptionally upregulates the downstream targets like BH3, PUMA, and NOXA. Further, PUMA and NOXA translocate the BAX and BAK (BCL-2 family members) to the mitochondria of oocytes and promote the release of caspase 9 and apoptotic proteins to trigger cell death. Whereas decreased expression of LhX8, VDAC2 leads to overexpression of autophagy pathway proteins that induce autophagy-dependent oocyte death

mTOR signaling is controlled by many factors like genotoxic stresses, osmotic stresses, metabolic stresses, and growth factors mTORC1 is sensitive to rapamycin and activates primordial follicle by phosphorylating and activating S6K1 through deactivation of eIF4E-binding proteins (4E-BPs) (Fig. 4.6) (Fingar and Blenis 2004; Wullschleger et al. 2006). S6K1 then phosphorylates and activates rpS6 leads to an increase in the protein translation (Fig. 4.6) (Wullschleger et al. 2006). Tuberous sclerosis proteins 1(Tsc1), Tuberous sclerosis proteins 2(Tsc2) complex, a negative regulator of mTORC1, helps to preserve the pool of dormant primordial follicles. Both Tsc and Phosphatase tensin homolog (PTEN) help in sustaining the quiescence of primordial follicles and act interdependently by downregulating S6K1 activity (Fig. 4.6) (Adhikari et al. 2009a; Reddy et al. 2009). This result was backed by a study where a mutation in the Tsc2 gene leads to the hyperactivation of the primordial pool due to overactivation of mTORC-1 activity resulting in premature ovarian failure (Adhikari et al. 2009b). 3-Methylcholanthrene (3MC), a potent ovotoxicant, causes follicle atresia and irregular early follicle activation via the PI3K/Akt and mTOR signaling pathways (Wullschleger et al. 2006). Drugs like AMP-activated protein kinase (AMPK) inhibitors and liver kinase B1 (LKB1) either activate or restrain primordial follicle activation by manipulating mTOR signaling (Lu et al. 2017; Jiang et al. 2016). The contribution of mTOR in oocyte atresia is also backed by studies in cyclophosphamide and cisplatin-induced ovarian dysfunctional animal models where inhibitors of mTOR can prevent premature ovarian failure (Fig. 4.6) (Goldman et al. 2017; Zhou et al. 2017; Tanaka et al. 2018).

4.5.2 TAp63α Guardian of Female Reproduction

Mammalian oocytes can live up to several decades and are highly likely to experience DNA damage. The inefficient DNA damage detection or removal of the damaged oocyte from the reserve pool may result in embryonic death or congenital disorders. In mice fetal ovary, primary oocytes appear to employ p53 to trigger apoptosis in response to DNA damage (Ghafari et al. 2009). After birth, the constitutive expression of TAp63α (the trans-activating isoform of p63) in primordial follicles makes them extremely sensitive to DNA damage. Like somatic cells, whenever an oocyte encounters DNA damage, the DNA damage response (DDR) in oocytes gets activated. The active DDR signals help repair the DNA or send the oocyte to death (Suh et al. 2006; Livera et al. 2008). DDR signaling is inducted by ATM/ATR kinases that phosphorylate downstream targets γ- H2AX to amplify DDR signaling, which activates effector kinase Chk-2 (Carroll and Marangos 2013). Then Chk-2 phosphorylates downstream targets, including TAp63α on Ser582, which facilitated the phosphorylation of the remaining serine residues by Casein Kinase 1 (CK1) (Fig. 4.6) (Tuppi et al. 2018). These sequential Phosphorylation events lead to irreversible tetramerization of Tap63α. Further, tetramerized Tap63α acts as a transcription factor for the p53 family response genes to promote either damage repair or programmed cell death (Fig. 4.6) (Kim and Suh 2014). Whereas in uncompromised

oocytes, Tap63α will be kept in an inactive conformation, i.e., in a dimeric state (Fig. 4.6). However, most of the existing mechanisms are drawn from mouse studies. There are no mechanistic insights available with livestock species. Therefore, it is necessary to investigate the oocyte DDR and repair mechanisms in livestock species to enhance fertility.

4.6 Death Mechanisms During Oocyte Atresia

The continuous decrease of the oocyte population during all the stages in the ovaries shows the hallmarks of programmed cell death (PCD). Majorly three types of PCD are observed in oocytes. Type-I PCD shows the characteristics of chromatin compaction, change in the cell morphology like shrinkage, contraction, and membrane blebbing followed by nuclear destruction. Type-I PCD involves both mitochondria-mediated (intrinsic) and surface death receptor-mediated (extrinsic) pathways. In the intrinsic pathway, increased ROS levels in mitochondria promote the change of the mitochondrial membrane potential through Bax/Bcl2. Further, the expression of Bax/Bcl2 triggers the cytochrome-c release in the cytoplasm, which in turn activates the downstream caspases 9 and 3. Then active caspase 3 cleaves the structural and regulatory proteins, including poly-ADP ribose polymerase-1 (PARP1), which commences the morphological and biochemical changes that lead to the death of the oocytes (Fig. 4.6). Unlike the intrinsic pathway, the extrinsic pathway is prompted by the coupling of the proapoptotic ligands FASL and TNFα to their receptor, which activates the downstream caspases. Further, the active caspases that cleave the regulatory proteins result in DNA wreckage followed by oocyte death (Fig. 4.6). Apoptotic pathways execute a crucial part in the longevity of the ovarian reserve. In mice, expression of Bcl2 correlates with healthy ovarian reserve (Morita et al. 1999; Flaws et al. 2001) and, opposingly, Bax mutant has shown increased primary and preantral follicles (Fig. 4.6) (Greenfeld et al. 2007). Also, initiator caspase 9 eliminates oocytes with prophase I meiotic errors through mitochondrial-mediated apoptosis (Ene et al. 2013). It has been noted that mice lacking Caspase 2 treated with doxorubicin have a more number of oocytes than wild-type controls (Bergeron et al. 1998).

Type-II cell death is called autophagic cell death, where targeted cytoplasmic components are carried to lysosomes for degradation by a double-membrane vesicle known as an autophagosome. In oocytes, autophagy plays a dual role; on the one hand, it helps in cell survival by promoting adaptive stress response; on the other hand, it induces cell death (Huber et al. 2012). In mice, the transcription factor Lhx8 in the oocytes was connected with the germ cell-specific gene Nobox. Lhx8 is also associated with follicle survival and maturation as the knockout of Lhx8 blocks oocyte growth beyond the primordial follicle stage. The increased expression of various autophagic markers such as LC3B, Beclin1, and ATG7 in the absence of Lhx8 in mouse oocytes indicates the autophagy-dependent death in all stages

(D'Ignazio et al. 2018) (Fig. 4.6). Also saw similar results in teleost fishes and Nile tilapia where autophagic regulation through the Beclin1-Bcl2 axis plays a vital part in follicular survival (Sale et al. 2019). Autophagy in the ovaries is also governed by Voltage-dependent anion-selective channel protein (VDAC2). VDAC2 inhibits the participation of autophagy in the regulation of follicular development by increasing the cooperation between Beclin-1 and Bcl-2-like 1 (BCL2L1) (Fig. 4.6). VDAC2 also enhances fecundity in the females by supporting the development of dominant follicles into a mature egg, which is crucial for oogenesis (Yuan et al. 2015). The oocyte quality is augmented with age through the daf- 2 IIS receptor mutation by Cathepsin B activity in *Caenorhabditis elegans* (Templeman et al. 2018). The expression of ATG14 and LC3 is increased by Melamine in oocytes, resulting in abnormal mitochondria and autophagy distribution, leading to oocyte death (Duan et al. 2015) (Fig. 4.6). Increased autophagy in granulosa cells results in the death of preantral follicles, whereas apoptosis in granulosa cells is the reason for antral follicular atresia. This result was supported by hyper ATG7 and cCASP3 signals in granulosa cells of preantral atretic follicles compared to the atretic follicles (Meng et al. 2018). The role of autophagy-mediated death was strengthened by the use of autophagy inhibitor 3-Methyladenine (3MA) along with apoptosis inhibitor Z-VAD-FMK(ZVAD), where they found a similar level of germ cell acidic vacuoles in only 3MA and combined treatment of 3MA and ZVAD (Rodrigues et al. 2009). Further, the role of autophagy to maintain oocyte population is evident as the knockout of Beclin1 and ATG7, two major regulators of this process, resulted in fewer oocytes after birth in mammalian models (Gawriluk et al. 2011). In addition, follicle numbers increased by the administration of Tat -beclin D-11 along with the increased expression of LC3-II/LC3-I (Watanabe et al. 2020). Type-III cell death is necrotic cell death, where the quick loss of plasma membrane structure and spillover of the intracellular components is prominent. Necroptosis is a cellular response to the increased stress caused by inflammation or infection. Necroptosis is initiated by the formation of TNFR complex I through TNF-α binding to its receptor. This interaction allows the loading of other TNRF related factors including, a cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, and receptor-interacting protein kinase 1 (RIP1), RIPK3, to form a necrosome. Successively phosphorylation of mixed-lineage kinase domain-like protein (MLKL) by RIPK3 results in the formation of a pore-forming oligomer to rupture the plasma membrane, leads to death via necroptosis (Vandenabeele et al. 2010). Necroptosis can also be caused by Toll-like receptors, NOD-like receptors, and in response to DNA damage. Emerging evidence in cows and humans suggests that a high level of cortisol and oxidative stress induces granulosa cell death leads to the low supply of nutrients, growth factors, and hormones required for follicle growth (Chaudhary et al. 2019). Because of starvation, ultimately, oocytes undergo death via necrosis. Also, observations in mice suggest reduced ovarian reserves, follicle maturation, and ovary development due to the increased TNF-α and immune suppression (Bagavant et al. 1999; Osnes et al. 2013; Shepel et al. 2014).

4.7 Biotechnological or Pharmacological Interventions to Delay the Ovarian Aging

Ovaries undergo aging quickly compared to other organs. The prime reason for ovarian aging is a faster decline in the quantity and quality of oocytes. As discussed above, ovarian aging could result from age, nutrition, physiological, genotoxicity, and chemotoxicity. Therefore, novel biotechnological approaches or pharmacological interventions are necessary to delay ovarian aging. Also, quantification of oocyte reserve is crucial before an animal is subjected to intervention. Thus, the development of cost-effective, easy oocyte quantification methods is necessary.

4.7.1 Quantification of Ovarian Reserves

Anti-Mullerian hormone (AMH) is a peptide hormone predominantly secreted by ovarian granulosa cells of preantral and antral follicles. The decrease in follicle numbers, particularly in growing follicles, correlates with a reduction of AMH levels. AMH can be measured throughout the reproductive cycle with approximate values between 0.2 and 1.26 ng/ml to distinguish the poor responders with approximately 85% sensitivity compared with ultrasound (Jirge 2011) in humans. In adult cattle, the AMH levels are constant throughout the reproductive cycles and positively correlate ($r = 0.90$) with morphologically healthy follicles. Also, AMH levels are heritable and similar in most breeds like Holstein, Nelore, European, and Zebu cattle. In other animals like mares, the ovarian reserves positively correlate with AMH values in the plasma. Other than goats and sheep, most of the farm animal AMH levels indirectly indicate ovarian reserve. In cattle, goats, and sheep, AMH levels and antral follicle count are practiced as diagnostic markers before superovulation in assisted reproductive technologies. Existing AMH quantification methods like ELISA are expensive and time-consuming. Therefore, the development of novel, inexpensive AMH quantification assays would be advantageous to assess the ovarian reserve in the livestock sector (Monniaux et al. 2014). Other than AMH, basal follicle-stimulating hormone (FSH), Inhibin B, basal estradiol, clomiphene citrate challenge tests are practiced to check the ovarian response in humans. Still, none of them can stand with the efficiency of the AMH test.

4.7.2 Suppression of Oocyte Death and Activation to Increase the Follicular Reserve

Oocyte numbers decrease with the rise of the mother's age, subsequently leading to reproductive failure. This reproductive failure is widespread in all domestic animals. For example, a cattle's life span is approximately 15 years, but many of them

show poor reproductive capabilities from 7 to 8 years. These animals are either slaughtered or strayed. These conditions severely affect the economy of the farmers. An increase in 2 or 3 lactations would benefit the farmers. Therefore, novel strategies are needed to increase fertility lifespan. The best possible plans are (i) protecting the oocytes from death, which might compromise the oocyte quality. (ii) maintain the oocytes for more extended periods in the early stages by slow activation. In mice, pharmacological inhibition of the mTOR by rapamycin extends the ovarian lifespan by limiting the over-activation of oocytes during chemotherapy (Goldman et al. 2017). Further short-term therapy with rapamycin increased the ovarian life in mice (Dou et al. 2017). In vitro treatment of bovine oocytes with 1 nm rapamycin showed the highest maturation rates (95.21 ± 4.18%), blastocyst formation (Pawel Kordowitzki et al. 2020). Like bovine, pig oocyte does not show any difference in maturation, but blastocyst formation rates are increased compared with controls in morphologically good and poor oocytes (Lee et al. 2015). Also, rapamycin rescues the inadequate developmental capacity of aged oocytes in pigs (Lee et al. 2014). In mice, the CHK2 inhibitor proved effective in recovering the primary oocyte from γ-irradiation through inhibiting the Tap63α phosphorylation. Re-implanting these ovaries into a sterile host regained fertility (Rinaldi et al. 2020). Like CHK2 inhibitors, ATM and CK1 inhibitors can be employed as ovarian life extenders (Tuppi et al. 2018). These inhibitors effectively work on germ cells; however, we cannot rule out the negative impact on somatic cells. Therefore, the development of novel drug delivery methods at the ovary is very much required.

4.7.3 Reactive Oxygen Species Increase Because of the Oxidative Stress

The increase in reactive oxygen species (ROS) impacts the oocyte reserve and quality. Also, ROS increase in follicular fluid of aged females, unfertilized eggs, and poor-quality embryos. Therefore, antioxidants have been utilized as anti-ovarian aging agents. Recently, pharmacological anti-aging agents or drugs gained importance in infertility treatments. Especially anti-ROS agents like Vitamin C, E, N acetyl L cysteine, CoQ, proanthocyanidin, quercetin, and curcumin are the most prevalent compounds used in infertility treatments (Ahmadi et al. 2016). For example, in chicken, Grape Seed Proanthocyanidin extends the ovarian lifespan by inhibiting oxidative stress (Liu et al. 2018). Melatonin improves follicle growth and survival rate by enhancing the antioxidant enzymes in laying hens (Hao et al. 2020). Further, studies in livestock species must choose the most effective method to protect the ovarian reserve.

Reconstituting complete gametogenesis *in vitro* from primordial germ cells is an important goal for reproductive biology and regenerative medicine. In female mammals, a minority of oocytes survive to enter the ovarian reserve, and only very few mature oocytes are available for fertilization. Achievement of a complete *in-vitro*

oogenesis system, which is fertilizable, will provide a unique tool to improve the fecundity and fertility in female mammals. The Obata lab recently reconstituted mouse oogenesis *in-vitro* starting from endogenous primordial germ cells (PGCs) and primordial germ cell-like cells (PGCLCs) derived from ESCs, or iPS cells (Hayashi et al. 2017). This discovery opens up a new era of reproductive biology research. Still, many questions remain unresolved, such as the low efficiency of PGCLCs differentiation compared to endogenous PGCs, the quality of the offspring, and most importantly, the applicability of such systems to livestock or human reproduction. However, using novel biotechnology approaches like 3D printed ovaries in exploiting the methods learned from mouse studies to recapitulate *in vitro* oogenesis using livestock cells would be beneficial in the livestock sector (Laronda et al. 2017).

4.8 Quality Control During Oocyte Maturation

Oocytes will be in a quiescent stage with a meiotic arrest with maintained cAMP levels. Further, drop-in cyclic guanosine monophosphate (cGMP) leads to meiosis resumption (Driancourt and Thuel 1998). The C-type natriuretic peptide (CNP)/natriuretic peptide receptor 2 (NPR2) pathway participates in the maintenance of meiotic arrest in mouse (Zhang et al. 2010), pig (Santiquet et al. 2014), cattle (Franciosi et al. 2014; Soares et al. 2017; Botigelli et al. 2018; Xi et al. 2018), and goat (Zhang et al. 2015). In mice, the binding of CNP to its receptor NPR2 in the cumulus cells stimulates the generation of cGMP. Further, cGMP relocated to the oocyte (Richard and Baltz 2014) and inhibits PDE3A activity (Vaccari et al. 2009), which in turn blocks cAMP degradation and preserves oocytes in the germinal vesicle (GV) stage (Zhang et al. 2010). In cattle, NPR2 is present in the oocytes (Botigelli et al. 2018); moreover, therapeutic strategies using CNP as a non-pharmacologic meiosis-arresting agent have shown the delayed meiosis restoration and increased the developmental capability of cattle oocytes (Soares et al. 2017; Botigelli et al. 2018).

Oocyte maturation is one of the final steps during oocyte development needed for proper fertilization and embryo development. In livestock, it starts after puberty with a transient increase in follicle-stimulating hormone (FSH). Successively, dictyate stage oocyte re-enters into meiosis under the influence of gonadotropins, FSH, and luteinizing hormone (LH), and complete metaphase II just before ovulation. It consists of two interlinked complementary processes; cytoplasmic and nuclear maturation. In addition to natural ovulation, mature oocytes also are obtained by superovulation or in-vitro maturation (IVM) (Singh et al. 2017). IVM offers the opportunity to utilize biological resources for large-scale embryo production from many decades in livestock. However, it is a multifaceted process requiring much more than nuclear maturation after the LH surge or after the discharge of the matured oocytes from its follicle. During IVM, the collected germinal vesicle (GV) stage

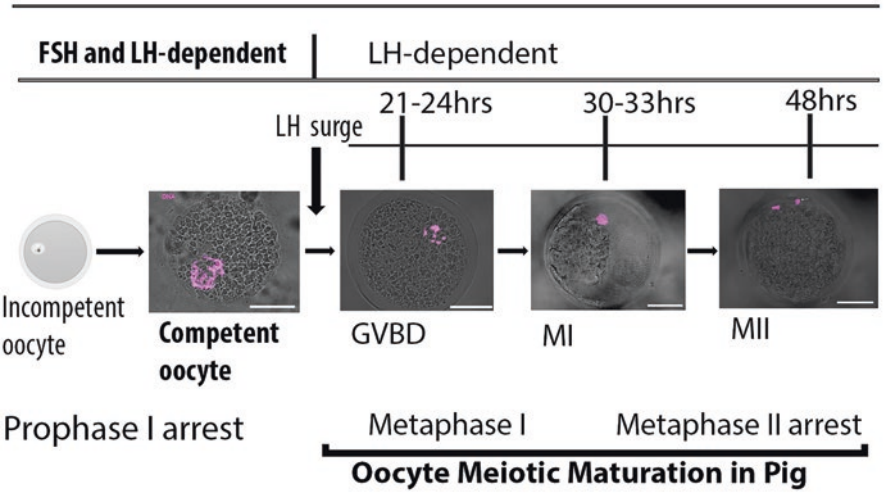

Fig. 4.7 Porcine oocyte meiotic maturation. During the estrous cycle, LH surge stimulates the maturation promoting factor (MPF) to start the resumption of meiosis. Oocyte maturation is a highly regulated process in which GVBD is the first visible sign of meiotic progression, followed by homolog chromosome segregation and the first polar body extrusion. Next, oocytes undergo a second meiotic division which is like a mitotic cell division. Later matured oocytes remain arrested in the MII stage until fertilization. Arrows indicate the polar body DNA. Scale bars = 50 μm

oocytes develop synchronously with the help of hormones (Figs. 4.1 and 4.7). The dictyate stage oocyte takes almost a similar time interval to reach metaphase II, either cultured in vitro or stimulated in the ovary by LH (Figs. 4.1 and 4.7) (Edwards 1965). However, oocytes isolated from less developed follicles have less developmental competence, mainly due to cytoplasmic maturation. It includes organelle redistribution, micro-and macromolecular changes. Here, we will discuss quality determining factors of oocytes during in vivo and in-vitro maturation.

4.8.1 Chromatin Configuration During the Oocyte Development

The initial belief is that smaller follicles have low developmental competence in terms of blastocyst formation. Also, oocytes aspirated from early antral follicles (0.5–2 mm in diameter) have a less compact chromatin arrangement than follicles size around 2–8 mm. In cattle, four chromatin configurations have been observed, namely GV0 to GV3. In the GV0, the chromatin looks mostly uncondensed and spread throughout the nucleoplasm. The highest degree of compaction was observed

in the GV3 level. Where chromatin was condensed into a single clump and located in a limited area of the nucleus (Figs. 4.1 and 4.7) (Lodde et al. 2007). In mice, chromatin reshapes from the non-surrounded nucleolus (NSN) shape to the surrounded nucleolus (SN) shape, in which chromatin spreads around the nucleolus as a ring (Mattson and Albertini 1990). A similar chromatin configuration was recognized in human oocytes, where chromatin configuration changes from spread to a dense structure surrounding the nucleolus (Combelles et al. 2003) with two intermediate stages (Sánchez et al. 2015). These chromatin transient structures indicate the stages of oocyte differentiation associated with different functional characteristics. For example, in bovine oocytes, the GV0 to GV3 chromatin structures correspond to an increase in transcription silencing (Lodde et al. 2008), alterations in epigenetic signs such as histone modification (Lodde et al. 2009), changes in nuclear, and cytoplasmic architecture (Lodde et al. 2008). Besides, these chromatin configurations from dispersed to compacted stages indicate the oocyte developmental competence (Lodde et al. 2008). Similar views were reported in humans and mice (Sánchez et al. 2015). However, how these chromatin variations modulate the cellular functions in the oocytes remains unexplored.

4.8.2 Cumulus Cells

In the follicle, oocytes grow in a highly coordinated manner with the help of surrounding somatic cells to acquire complete developmental competence. The cumulus cells are a layer of closely linked granulosa cells, develop into a cumulus-oocyte complex (COC). Gap junctions are involved in the cross-talk between the cumulus cells and the oocyte in the COC complex. Gap junctions are actively participated in channeling the metabolites and nutrients through paracrine factors. Also, gap junction-mediated communications with the oocyte help in germinal vesicle breakdown and succession to metaphase II (Moor et al. 1980; Eppig 1982; Furger et al. 1996). During *in-vitro* maturation, the expansion of cumulus cell layers is directly linked to the oocyte quality (Cetica et al. 1999; Auclair et al. 2013). Removal of cumulus cells affects the oocyte maturation in rats (Vanderhyden and Armstrong 1989), cattle (Chian et al. 1994; Zhang et al. 1995) and pigs (Wongsrikeao et al. 2005). Further, oocyte and COC co-culture strategies have been observed to partly restore the developmental competence of denuded oocytes in cattle (Hashimoto et al. 1998; Luciano et al. 2005) and mice (Cecconi et al. 1996; Yamazaki et al. 2001). Also, oocyte quality will be judged based on the surrounding cumulus cell layers thickness and compactness. “A and B” grade oocytes have better development competency in comparison to “C” and “D” grade oocytes (Yang and Lu 1990; Madison et al. 1992; Hawk and Wall 1994; Hazeleger et al. 1995; Khurana and Niemann 2000; Bilodeau-Goeseels and Panich 2002).

4.8.3 Mitochondria

Oocyte maturation is a dynamic process in which mitochondria-generated ATP plays a crucial role in nuclear and cytoplasmic events (Dalton et al. 2014; Krisher and Bavister 1998; Stojkovic et al. 2001; Van Blerkom 2004, 2011; Van Blerkom et al. 1995). One of the apparent indicators of cytoplasmic maturation is a considerable increase in the oocyte size (Figs. 4.1 and 4.7). In this phase, the developing oocyte undergoes local changes in cytoplasmic organelles such as mitochondria, endoplasmic reticulum, ribosomes, cortical granules, Golgi complex, etc. Recent observations suggest that mitochondrial distribution in the cytoplasm is crucial during oocyte maturation (Wilding et al. 2001; Sun et al. 2001; Dumollard et al. 2004; Zhang et al. 2008; Eichenlaub-Ritter et al. 2011; Bianchi et al. 2015). In mice, mitochondria aggregate during the GV stage and disperse throughout the cytoplasm at G2/M transition. Then during the polar body extrusion, all the mitochondria will be redistributed into the entire area of the cytoplasm (Van Blerkom and Runner 1984; Dumollard et al. 2006).

Besides this, mitochondria are actively associated with microtubule assembly and disassembly during meiotic spindle formation, and its dysfunction is one of the most common causes of infertility in aged animals. Recent reports suggest that supplementation of nicotinamide mononucleotide (NMN), a nicotinamide adenine dinucleotide (NAD+) metabolic precursor, restores oocyte quality in old animals (Bertoldo et al. 2020). In addition, oocyte quality is diminished during postovulatory aging because of the overproduction of ROS and concomitant depletion of the antioxidant system leading to oxidative stress (Goud et al. 2008).

4.8.4 Biotechnological Interventions in Oocyte Maturation

Considering the importance of oocyte quality in livestock and humans, various biotechnological and pharmacological interventions have been examined in the last few decades to enhance the quality of mature oocytes. One such approach is an addition of FSH-primed new granulosa cells in the co-culture systems and found significant improvement in blastocyst formation rate (Schramm and Bavister 1996). In another approach, buffalo oocytes co-cultured with cumulus cells could improve the developmental competence of denuded oocytes in IVM conditions (Feng et al. 2013). A similar approach produced higher efficiencies of the first polar body formation in porcine oocytes (Ju and Rui 2012). In addition to the co-culture strategy, the use of cumulus cells adapted medium also improved the IVM outcome (Madkour et al. 2018; Abdel-Ghani et al. 2011). Recent findings suggest that mitochondria-related issues can be fixed by mitochondrial replacement therapy (MRT), where healthy embryos and live birth of monkeys were achieved by transferring the first polar body (PB1T) to replace the abnormal mitochondria (Wang et al. 2020).

The age of the animal is also an influential factor that regulates the quality of the mature oocyte. Several biotechnological interventions have been explored in the last few decades to counter the age-dependent oocyte quality. The most well-known methods, including the substitution of an ooplasm, germinal vesicle, pronucleus, and spindle chromosome, are considered. However, mitochondrial heteroplasmy is a primary concern in these techniques.

4.9 Impact of Ovarian Disorders on Livestock Follicle Reserve and Quality

The ovarian disorders and their influence on the economy, especially in the dairy sector, are renowned (Lopez-Gatius et al. 2002; Tanaka et al. 2008; Roman et al. 2020). Dawson and his colleagues found that inflammatory lesions in the uterus, fallopian tubes, ovarian bursae, and ovarian disorders like cystic ovaries are the major reasons for the sterility in the cows (Dawson 1958, 1963). Therefore, avoiding or treating these problems is very important in livestock reproduction. Ovaries are the major female reproductive organs responsible for folliculogenesis and oocyte maturation (Fig. 4.6). Dysfunctions in ovaries lead to various clinical or subclinical diseases, including cystic ovarian syndrome 6–19% in cows (Ijaz et al. 1987) and up to 50% in female dogs (Akihara et al. 2007), 5–10% in buffalos (Abalti et al. 2006; Ali et al. 2009; Purohit 2015), 40–50% endometriosis in buffalos and cows (Katkiewicz et al. 2011; Al-Dori et al. 2020), and 15–20% premature ovarian failure and tubule blockage in humans (Richards and Pangas 2010; Aviel-Ronen et al. 2014; Nahum-Shani et al. 2015; Pavone and Lyttle 2015) which are causing permanent infertility.

An ovarian cyst is characterized as a persistent fluid-filled cavity that can grow larger than 25 mm diameter, affecting the estrous cycles (Teshome et al. 2016) (Fig. 4.8). In cattle and other animals, mainly three types of cysts are present: follicular cysts, luteal cysts, and cystic corpus luteum (CL). Sometimes cystic CL (fluid-filled cavity) structures that appear after ovulation look similar to the other two cysts (Vanholder et al. 2006); however, CL differs clinically from the follicular and luteal cysts. In contrast, follicular cysts and luteal cysts are etiologically and pathophysiologically related but differ clinically. Some cows develop ovarian cysts that will be automatically resolved. At the same time, others develop a large non-steroidogenic cyst that affects the animal's fertility (Vanholder et al. 2006). A sequence of endocrine changes drives ovulation during the estrous cycle. During the follicular phase, serum estrogen levels are increased by endocrine signaling in granulosa cells (Laven 2019). The increased estrogen induces a transitory surge in Luteinizing Hormone (LH), which persuades the ovulation usually in 14 h (mouse), 27 h (goat), 24 h (humans). It lasts up to 48 h, depending on species. Further, the follicles undergo rupture followed by oocyte release to form the corpus luteum (CL). Then granulosa cell synthesized progesterone helps nurture the oocytes and

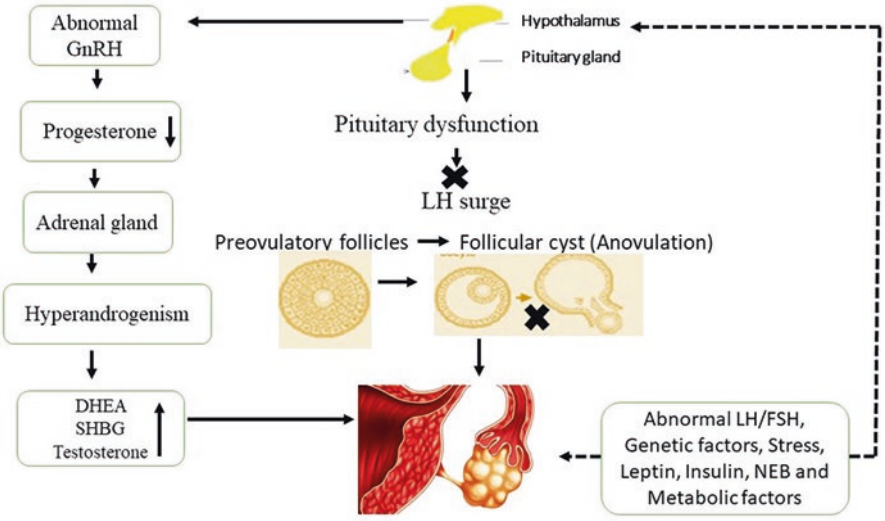

Fig. 4.8 Mechanism of cystic ovaries. In cystic ovarian disease, dysfunction of the pituitary alters the release of LH (LH surge) under the impact of oestradiol. After that, the dominant follicle fails to ovulate and results in anovulation, resulting in altered levels of estrogen, progesterone, FSH, and LH. At the same time, the androgen levels increase than usual with the drop in estrogen and progesterone. In addition, genetic factors, insulin resistance, negative energy balance (NEB), leptin, and metabolic factors interfere with the hormonal axis and lead to the etiology of COD in cows

maintain the uterine lining; consequently, fertilized oocyte can implant or attach to the uterus (Tsutsumi and Webster 2009). Progesterone levels peak about 7 days afterward the LH surge, which helps implantation (Kumar et al. 2013). Approximately 14 days after maturation or ovulation, the body begins the process of menstruation (in humans). The estrous cycle (in animals) includes clearing out the uterine lining and the unfertilized oocyte so the ovaries can start again. But in cystic ovaries, follicles remain in the primary or secondary stage with no follicle rupture (ovulation), leading to anovulation. These follicles further continue for more than 60 days, which leads to an enlargement of the growing follicles from 25 to 60 mm in diameter (Hancock 1948; Vanholder et al. 2006). In bitchs follicular cysts persist more than 21 days, or proestrus will continue for more than 40 days (Kumar et al. 2019a, b). Despite decades of research on ovarian cysts because of the diversity, time of appearance, and clinical symptoms, the exact cause of pathogenesis is still unknown (Weber et al. 1998; Lummaa 2003). One of the primary characteristics of ovarian cysts compared to luteal cyst is low circulating progesterone concentration (Kesler and Garverick 1982). Based on the available literature, it is considered that several factors contribute to the etiology of cystic ovaries, like genetic, phenotypic, metabolic diseases, negative energy balance (NEB), high productivity, retained placenta, dystocia, stress, and environmental factors in animals as indicated in (Fig. 4.8) (Kesler and Garverick 1982; Vanholder et al. 2006). In humans, it is described by

high levels of androgens, insulin, LH, ovulation dysfunction, abnormal growth of hair on body parts, and sterility (Franks 1995; Moran and Teede 2009; Toulis et al. 2009). Reduction of oocyte quality and embryonic developmental competence in cystic ovarian diseases are associated with irregular hormonal, metabolic functions, and alterations in the intrafollicular environment (Abbott et al. 2008; Dumesic and Abbott 2008). Altered male hormone concentrations in the follicular fluid (FF) are associated with raised serum LH levels, which may inhibit the growth of the dominant follicle and follicle death (Billig et al. 1993; Kurzawa et al. 2008). Further, a rise in LH levels probably suppresses FSH function, resulting in dysfunction of granulosa cell (GC), subsequently leads to aberrant follicular maturation and oocyte quality (Tarlatzis and Grimbizis 1997; Dumesic et al. 2002; Tesarik et al. 2003; Van der Spuy and Dyer 2004; Wood et al. 2007). Besides, increased LH induces oocyte apoptosis by affecting follicle release and nuclear maturation (Yoshimura and Wallach 1987; Kurzawa et al. 2008). Also, abnormal endocrine functions in meiosis result in chromosomal abnormalities and impaired first polar body extrusion (Sengoku et al. 1997) possibly contributes to embryonic aneuploidy in PCOS women (Weghofer et al. 2007). Recent observations suggest that exposure of premature oocytes to higher LH levels diminishes the oocyte and embryo quality which eventually leads to an increase in miscarriages in cystic disease females (Balen et al. 1993; Urman et al. 2004). Elevated androgens like testosterone are responsible for cystic ovaries. Also, androgens affect *in-vitro* maturation (IVM), fertilization, and embryo growth (Dumesic et al. 2007; Patel and Carr 2008). Testosterone injection in prenatal ewes resulted in a reduction of primordial follicles and a significant increase in remaining follicles (Steckler et al. 2005). In addition, hyperandrogenism influences oocyte maturation via altered calcium levels (Tesarik and Mendoza 1995, 1997; Jabara and Coutifaris 2003). Data from *in vitro* oocyte maturation studies in rodents (Eppig et al. 1998) and cattle (Galal and Mitwally 2009) suggest that insulin and FSH enhance the mRNA expression of LH receptor in granulosa cells (Dumesic et al. 2002; Diamanti-Kandarakis 2008). The increase in LH levels reduces the healthy quality eggs (Eppig et al. 1998; Dumesic et al. 2002, 2007) by constraining FSH-induced estrogen synthetase activity (Galal and Mitwally 2009). Hyperinsulinism possibly stimulates local androgen production, which results in compromised oocyte quality at the molecular level (Qiao and Feng 2011). Overexpression of insulin receptors in GC and theca cells (Dumesic et al. 2002, 2007; Kezele et al. 2002) alters several factors involved in the cell cycle, leading to a change in spindle architecture and centrosome function in the case of cystic oocytes (Wood et al. 2007). Altered levels of epidermal growth factor (EGF), an essential component of the oocyte microenvironment, inhibit the estrogen synthesis in GCs, which could be a possible cause of follicular detention in cystic ovaries (Almahbobi et al. 1996, 1998; Giovanni Artini et al. 2007).

Postpartum reproductive diseases constitute a significant threat and cause heavy economic losses to the dairy industry. These include uterine infections like endometritis, metritis, mucometra, and pyometra. These infections, delay in the onset of activities of the ovaries leading to systemic illness, loss of milk and meat production, and a marked drop in infertility. Out of these uterine infections, gynecological

inflammatory diseases like metritis and endometritis affect fertility and milk production in livestock species. The impact of these diseases, including reducing milk production, ranged between 2 and 13 kg/day during a period that may last for 2–20 weeks. Overall production loss per lactation ranges from 100 to 2000 kgs (Ettema and Santos 2004). Around 40% of animals develop these diseases within the 14 days of calving, and in 15–20% of these animals, the infection persists and leads to chronic uterine diseases like metritis and endometritis (Dohmen et al. 2000; Sheldon and Owens 2018). In India, the clinical surveys report that metritis and endometritis vary from 3% to 20% in buffaloes. Interestingly, 12% and 29.69% of crossbred cows show clinical and subclinical endometritis. Endometriosis not only causes infertility but also results in subfertility even after successful clinical resolution of the disease. Therefore, these diseases are described as a disease with a social and dairy care importance. However, no non-invasive diagnostic techniques are available to identify these complex diseases. Besides, contemporary treatment strategies are not adequate because of the poor efficiency, higher reversal rate, and practical challenges to examine these complicated diseases. Metritis is the inflammation of the uterus consisting of both the endometrial and the muscular layer. Most of the cases occur during the first 10–14 days of delivery, and sometimes it is referred to as toxic puerperal metritis. In contrast, endometritis refers to inflammation of the endometrium and the mucus membrane of the uterus without any systemic signs during 10–60 days of calving. The prevalence of postpartum endometritis and metritis depends upon the occurrence of early postpartum uterine diseases.

Altered ovarian signaling with slower follicle growth, anovulation, and irregular ovarian cyclicity is usual in cattle with uterine infections (Opsomer et al. 2000; Sheldon et al. 2002, Ribeiro et al. 2013). In most cases, even after treatment of uterine disease, cows were unable to conceive (Borsberry and Dobson 1989; LeBlanc et al. 2002). One of the main reasons for endometriosis is the imbalanced generation of pro and anti-inflammatory cytokines during the first postpartum week (Herath et al. 2009). Dickson et al. (2020) reported that bacterial-induced endometritis in Holstein cows resulted in increased expression of the endometrial inflammatory response and a mucopurulent vaginal discharge as compared to the healthy group. Further, they found that the bacterial infusion resulted in a 15% reduction of healthy morulae production than vehicle controls. Interestingly, endometrial expression of IL6 is inversely correlated with healthy oocytes. This data was supported by recent findings of Piersanti et al. (2019), where an infusion of bacteria (*Escherichia coli* and *Trueperella pyogene)* in heifers altered the oocyte transcriptome. Specifically, inflammatory/anti-inflammatory signaling pathways like interferon, HMGB1, ILK, IL-6, and TGF-beta were downregulated 4 days after infection and upregulated 60 days after post-infection. In addition, increased endometrial mRNA expression and serum pro-inflammatory factors were reported in cows with endometritis compared to healthy cows (Gabler et al. 2010; Galvao et al. 2011; Kasimanickam et al. 2014). Also, reports suggest that cytokines are involved in follicular development, oocyte release, CL formation, and regression (Sakumoto and Okuda 2004; Trombly et al. 2009; Singh et al. 2016). Taken together, the above pieces of evidence suggest that the inflammatory mediators released during endometriosis probably aid in

declining the oocyte quality. Recently Hanege et al. (2019) observed fewer oocytes in endometrial cyst than the ovary without any cysts. Also, they found a notable increase in the arrested oocytes at germinal vesicle (GV) and metaphase I (MI), in patients with endometriosis-associated infertility, compared to the control group (Dumesic et al. 2015). Elevated inflammatory ILs in endometriosis cases have been reported, which might interfere with the mechanism of spindle formation of egg development (Goud et al. 2014; Sanchez et al. 2017). Eggs from endometriosis females showed increased cortical granule loss and zona pellucida (ZP) hardening. This ZP hardening may affect the penetration of sperm into oocytes, probably through a reduced dissolution of the ZP, which leads to failure in fertilization (Goud et al. 2014). Therefore, to improve fertility in livestock, a profound understanding is required, how ovarian cyst and endometriosis affect the extra and intra-ovarian environment that modulates the oocyte development, maturation, and potential embryonic developmental competence.

4.9.1 Biotechnological Interventions in Ovarian Disorders

ART techniques like in-vitro fertilization (IVF) are extensively used to produce offspring in case of endometriosis and cystic ovarian in humans (Canis et al. 2001) and animals (Ijaz et al. 1987; Ishwar and Memon 1996). Intracytoplasmic sperm injection (ICSI) is an extensively used technique for infertility treatment in the horse-breeding industry (Allen 2005). Treatment strategies have been developed with GnRH and PGF2α to synchronize ovulation, follicular dynamics, and luteolysis. These strategies allowed fixed timed artificial insemination (TAI) of animals without examination of estrus and treatment of cystic ovaries in cattle. The use of human chorionic gonadotropin (hCG) is favored to induce ovulation in the case of the cyst. However, GnRH dosing is effective at 100 mcg and less antigenic than hCG. Prostaglandin (PG) F2α products were usually given a weekday after hCG or GnRH treatment to begin the estrous cycle (Amer and Badr 2008). Other than hormones, many plant-based natural products were used to treat cystic ovaries and endometriosis to avoid surgical and medical intervention. *Viburnum opulus (*Saltan et al. 2016), *Sea buckthorn, St. John's wort* (İlhan et al. 2016), and Black cohosh (Azouz et al. 2020) are some common herbal drugs used to treat cystic ovaries and endometriosis. However, systematic investigations are necessary to take them to the field level as drug molecules. Although, imaging techniques like ultrasound, MRI, and PET-CT provided the most definitive diagnostic way of endometriotic cysts in animals. However, proper biotechnology-based techniques and strategies are required to diagnose the oocyte quality in cystic ovaries and endometriosis.

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Chapter 5
Metabolomics of Food Systems

Sandip Kumar Patel, Bhawana George, Neeraj Jain, and Vineeta Rai

Abstract 'Food metabolomics' is an emerging area in metabolomics, enabling food processors and scientists to understand the biochemistry and composition of food with precision, speed, and efficiency. The approach is applied to identify food resources and nutrition biomarkers, organic and genetically modified food authentication, geographic origin screening, and elucidation of environmental stress feedback in food resources and livestock research, quantitate and quantify dietary intake and exposure, and provide insights into the molecular mechanisms underlying sensory and nutritional characteristics. Food metabolomics encompasses plant to human nutrition ranging from soil quality, food resources, food processing to human nutrition. Understanding the bioactive and nutritional content of the food is becoming an emerging area of metabolomics. Since it's an emerging area, there are several challenges: lack of optimized workflow, uncharacterized metabolites, and lack of databases. Typical food metabolomics workflow includes targeted and untargeted metabolomics analysis in conjunction with chemometric analysis. Food databases help to characterize 'unknown metabolites.' This book chapter describes the recent trends and application of food metabolomics.

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5.1 Introduction

Metabolomics deals with the systematic analysis of endogenous and exogenous small molecules (<1kD) involved in primary or secondary metabolic processes. Metabolomics is fascinating due to the vast diversity of the metabolites classified into amino acids, lipids, nucleotides, carbohydrates, organic acids, etc. The organism's metabolic repertoire changes consistently and significantly during growth and development and interactions with environmental factors. The metabolic shifts thus represent the physiological state of the organism and have a strong correlation to the phenotype. Metabolomics is applied to all arena of biological sciences ranging from human health to agriculture (Kim et al. 2016; Tian et al. 2016). Assessing food quality is a prime area of the food industry today since there is increasing awareness among consumers regarding food safety and composition. Food metabolomics is an emerging area in metabolomics, enabling food processors and scientists to understand the biochemistry and composition of food more quickly and efficiently. With the ever-increasing population, reduction in agricultural lands, climate change, and environmental pollutants, food metabolomics analysis is imperative for food security and human health. This chapter focuses on metabolomics workflows, applications, and challenges in agriculture, livestock, processed food, human nutrition, and plant resource food. New advances such as food metabolomics databases, the application of metabolomics to screen genetically modified (GM) crops, and organic food are also discussed. This chapter also highlights the challenges in the food metabolomics research, such as sample preparation, data analysis, identification of unknown compounds.

5.2 Metabolomics Analysis

5.2.1 Study Design

The study design of metabolomics analysis should be based on the biological question. Two different metabolomics approaches are employed based on the analysis requirement: untargeted and targeted (Fig. 5.1). If the research is hypothesis-driven, primarily to recognize the prior characterized and biochemically annotated metabolites, then a **targeted approach** is preferred. The analysis could be undertaken quantitatively or semi-quantitatively based on the standards. **Untargeted metabolomics** is ideal for hypothesis development, as it simultaneously identifies and quantifies several unknown/known metabolites. The identification of unknown

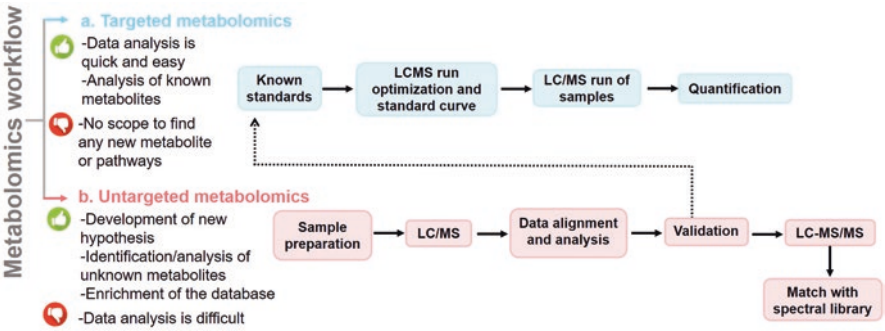

Fig. 5.1 Metabolomics workflows (**a**) targeted and (**b**) untargeted

features is performed by matching the metabolic MS/MS fingerprints with the public spectral library repository or standards, which leads to limited metabolite identification. Therefore, many potentially useful information in MS/MS data sets remains uncurated. Several *in silico* tools like Global Natural Product Social Molecular Networking (GNPS) could catalog the uncurated MS/MS data sets via a spectral correlation and visualization approach (Wang et al. 2016). Both methods have their advantages and limitations. There is no universal metabolomics workflow that is one-for-all due to vast metabolites complexity.

5.2.2 Sample Collection, Quenching, and Storage

Sample collection and storage are very critical. The metabolites are very dynamic; hence sampling time becomes essential, and samples should be collected systematically. Consistency is the key to sample collection, particularly for long-term experiments. Food habits, age, sex, social-economic status, geographic location, etc., should be considered for animal models or human subjects. Likewise, species, environmental factors like watering patterns, nutritional content, light, moisture, day-night cycles, and development stage should be accounted in plant study. The tissue/bacterial samples should be washed with buffer or water before storage to remove any media/external components. Likewise, for exometabolomics research, the samples should be filtered to remove any cells in the media. Further, samples may be spiked with the known concentration of metabolite "standard" to evaluate any degradation/change in the metabolite during storage. As soon as the samples are collected, they should be quenched with liquid nitrogen or solvents like methanol and stored at −80 °C until processed further.

5.2.3 Workflow

Typical metabolomics workflow (Fig. 5.2) includes
(i) **Study designing**, the most critical factor for any metabolomics study. Study design comprises but is not limited to asking the right question or hypothesis based on which sample size, controls, sampling time, metabolite extractions solvents/methods, analytical tools, data analysis, etc., are decided.
(ii) **Metabolite extractions:** the intracellular metabolite extraction goals include separation of the small molecules from cell debris or other cellular biomolecules like protein, nucleic acids, etc. For the exometabolites, like the extraction of metabolites from the soil, the aim is to separate metabolites from the complex matrix. The metabolite extraction approaches should be consistent and minimize the losses due to biochemical/photochemical conversion or degradation. However, the biases are inevitable due to the wide dynamic range of metabolites and varying solubility quotient (Phapale et al. 2020). A wide range of solvent choices is available based on metabolite interest and chemistry. Apart from the solvent, temperature, pH, and desired molecular weight of the metabolite of interest should be considered. Monophasic and biphasic extraction approaches are employed based on the metabolites of interest. In monophasic extraction, only one solvent is used, while for biphasic extraction, a combination of polar and non-polar solvents are used to achieve a comprehensive metabolite coverage. Samples may be spiked with the known concentrations of standards to normalize for metabolite losses during extraction. Again, the standard spiked could be stable isotope-labeled (absolute standards) or pseudo standards that are primary or secondary metabolite not present in the experimental sample. For example, reserpine, curcumin (found exclusively in plants) could be spiked in serum/blood samples. Likewise, process control or negative control is vital to eliminate any contamination from leached pipette tips, centrifuge tubes or mass spec peak tubings, etc. Last but not least, sample homogenization should be considered; based on our information on the spatial localization of the metabolites, which suggests different regions of the tissue might have different concentrations or compositions of metabolites. The extracted metabolites should be aliquoted to prevent any metabolite degradation due to freeze-thaw.
(iii) **Sample complexity could be reduced on-line or off-line.** For on-line metabolite separation, liquid/gas chromatography (LC/GC), capillary electrophoresis (CE), and ion-mobility spectrometry (IMS) (Fig. 5.3) are attached to the mass spectrometry.

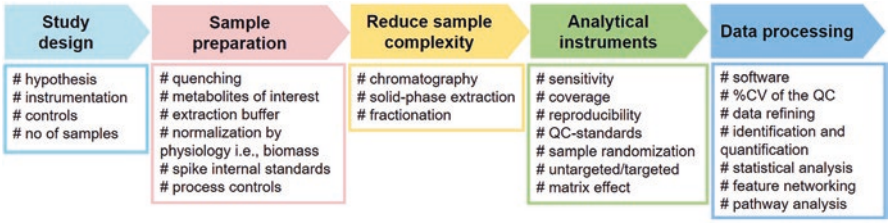

Fig. 5.2 Typical metabolomics workflow

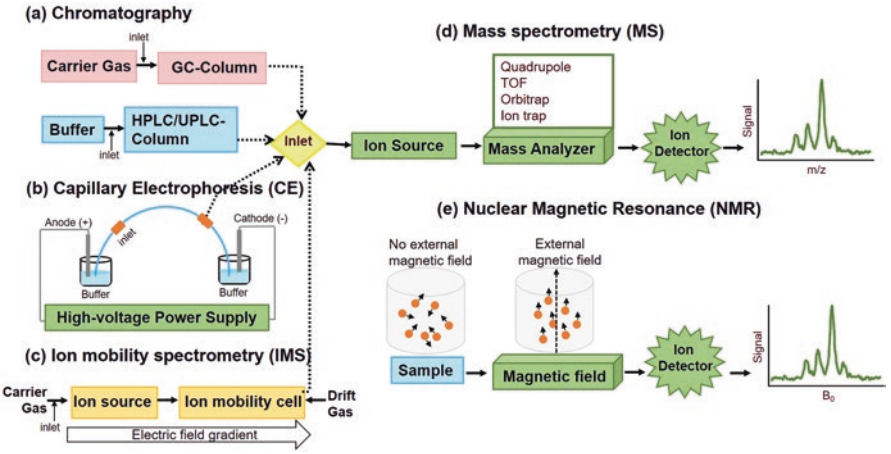

Fig. 5.3 (**a**) Chromatography, (**b**) capillary electrophoresis, (**c**) ion mobility spectrometry, (**d**) mass spectrometry, and nuclear magnetic resonance working principle

The most common off-line metabolite separation includes solid-phase extraction and LC-based fractionation based on the retention times. This step is essential in complex samples, where several masses co-elutes and make the data analysis challenging.

(iv) **Analytical instruments:** are selected based on experimental needs. For example, Gas-chromatography is employed to analyze the volatiles, or direct infusion mass spectrometry is used to analyze less complex metabolite extracts. GC uses gas (usually an inert gas or an unreactive gas) as a mobile phase and solid or liquid stationary phases. GC separation is highly robust as it separates metabolites based on vapourization temperatures. Thus, the technique is limited to the analysis (identification and semi-quantification) of molecules that are vaporized below 350–400 °C without decomposing or reacting with the GC components. Primarily to lower the vapor pressure, the metabolites are derivatized. LC uses liquid (acid, base, or neutral solvents) as a mobile phase and solid stationary phase. LC separation is highly variable and depends on the combination of mobile and solid phase, solvents, column properties like column material, length, diameter, pore size, temperature, etc. LC offers versatility and no or limited sample pre-processing requirement (Fig. 5.3a). CE separates ionized molecules in the liquid phase based on their electrophoretic mobility. The greater the electric field applied, the faster the mobility. Thus, the approach is inclusive for the analysis of ions (charged molecules) but not neutral species (Fig. 5.3b). IMS separates ionized molecules in the gas phase based on their mobility in a carrier buffer gas (Fig. 5.3c). Mass spectrometry (MS) measures the mass-to-charge ratio (*m/z*) of the ionized molecules. MS finds application in absolute or relative quantification of the metabolites, identify unknown molecules based on molecular weight and fragmentation patterns, a structure prediction. MS comprises three major components: ionization source, a mass analyzer, and an ion detector. The ionization source converts molecules to gas-phase ions, which could be negatively or positively charged. Hard and soft ionizations could be employed.

Hard ionization like Electron Impact ionization (EI) causes extensive fragmentation of the ions (ambiguous identification of the molecule weight) and is incompatible with LC, hence a method of choice for GC-MS. While soft ionization like electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption ionization (MALDI) has gained popularity and increased the MS applications in metabolomics analysis. A mass analyzer sorts and separates the ionized molecules according to mass-to-charge (m/z) ratios. Quadrupole (Q), Time-of-flight (TOF), Ion trap, Orbitrap, etc., are few examples are mass analyzers that are interchangeably used for metabolomics analysis based on the prerequisites for sensitivity, precision, accuracy, or resolution. A detector detects the sorted ions, and a mass spectrum/chromatogram is generated representing the m/z ratio against intensity/relative abundance (Fig. 5.3d). Each technique has advantages and limitations (Johnson and Gonzalez 2012). To reduce the complexity of the analysis, lower the sample volume and minimize analytical variation, several separation-free MS techniques like direct infusion-MS, MALDI-MS, mass spectrometry imaging (MSI), and direct analysis in real-time (DART)-MS are gaining popularity. Mass spectrometry imaging (MSI) has revolutionized MS-based metabolomics by providing spatial resolution. MSI is operated in two modes: imaging (Stoeckli et al. 2001) to correlate with histology and profiling to know the overall metabolites (Cornett et al. 2006). MSI technique is applied in broad areas of plant biology, including development, defense, and responses to abiotic and biotic stress, and the developing field of spatial-temporal metabolomics. Nuclear Magnetic Resonance (NMR) relies on detecting the electromagnetic signals generated due to the perturbation of the nuclei in a weak oscillating magnetic field (Fig. 5.3e). NMR finds application in quantifying the known compounds or identifies unknown compounds based on the match against the spectral libraries, or infer the basic structures. Chromatography or reducing the metabolite complexity is essential to reduce the matrix effect, identify reteition time that adds third dimention to the MS data, and enhance the MS/MS data quality.

(v) **Data analysis:** the aim of the data analysis is to get rid of the aritifacts, contanimants and redundant peaks, identify/quantify biologically relevant peak and statistical and pathway analysis. Metabolomics data analyis could be performed using propriety tools like mass propessional profiler (MPP), compound discoverer, etc., or open source tools like mzMine or XCMS. Metabolomics data analysis pipeline is shown in Fig. 5.4.

5.3 Food Metabolomics

'Food metabolomics'is the application of metabolomics in food systems, including processes from agriculture-to-human nutrition: (a) soil quality, (b) food resource, (c) food processing, and (d) human nutrition (Fig. 5.5).

Considering the constant change and evolution in the food supply and the ever-increasing and continuous launch of new products and formulations into the marketplace, there is a need to keep track of the vital food metabolites. Moreover, raw

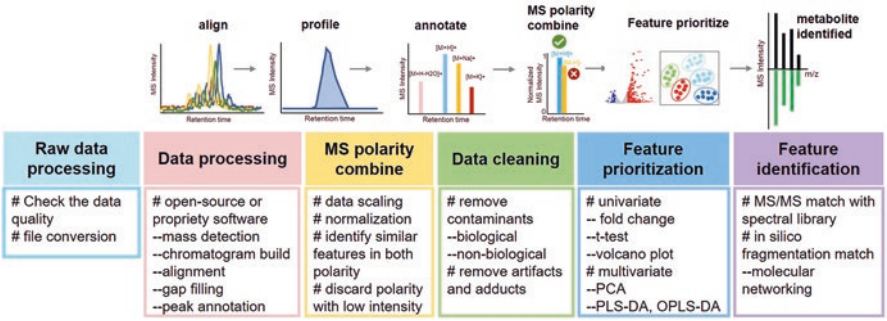

Fig. 5.4 Untargeted metabolomics data analysis workflow

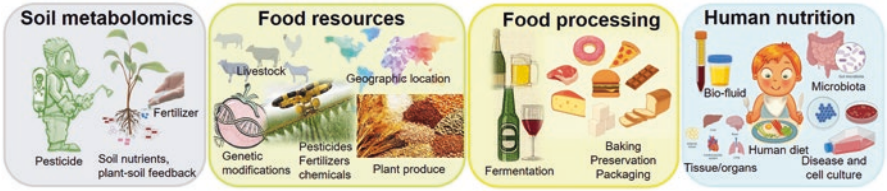

Fig. 5.5 Applications of food metabolomics

agricultural products, their composition like nutritional value, assimilated environmental contaminants, pesticides, etc., are also continually changing, leading researchers, nutritionists, and food policymakers to emphasize beyond single nutrients to develop holistic nutrition plans foods, food groups, and dietary patterns. Also, there is a growing urge for transparent and easily accessible information about nutrients and other food components. Typical workflow and challenges encountered to perform food metabolomics experiments are shown in Fig. 5.6.

Several natural and non-natural food metabolomics annotated databases are made available in the public domain (Table 5.1). Briefly, food databases are in place to get the list of possible metabolites, chemical and biological properties, structures, spectral data, related metabolic pathways, and their presence or concentration in food products based on the query m/z or elemental formula. Recently USDA launched an integrated, research-focused data system, 'FoodData Central,' that provides expanded data on nutrients and other food components and links to sources of related agricultural, food, dietary supplement, and additional information (https://fdc.nal.usda.gov/). The data in FoodData Central includes five data types, including Foundation Foods and Experimental Foods. In a similar effect, Dr. David Wishart Research Group at the University of Alberta annotated a comprehensive 28,000 food metabolite 'FooDB' (http://www.foodb.ca/) that comprises food constituents flavor, color, taste, texture, and aroma. Various European research groups have also taken the initiative to develop databases with tailored phytochemical components of the food metabolome. One such example is Phenol Explorer, which comprises 500

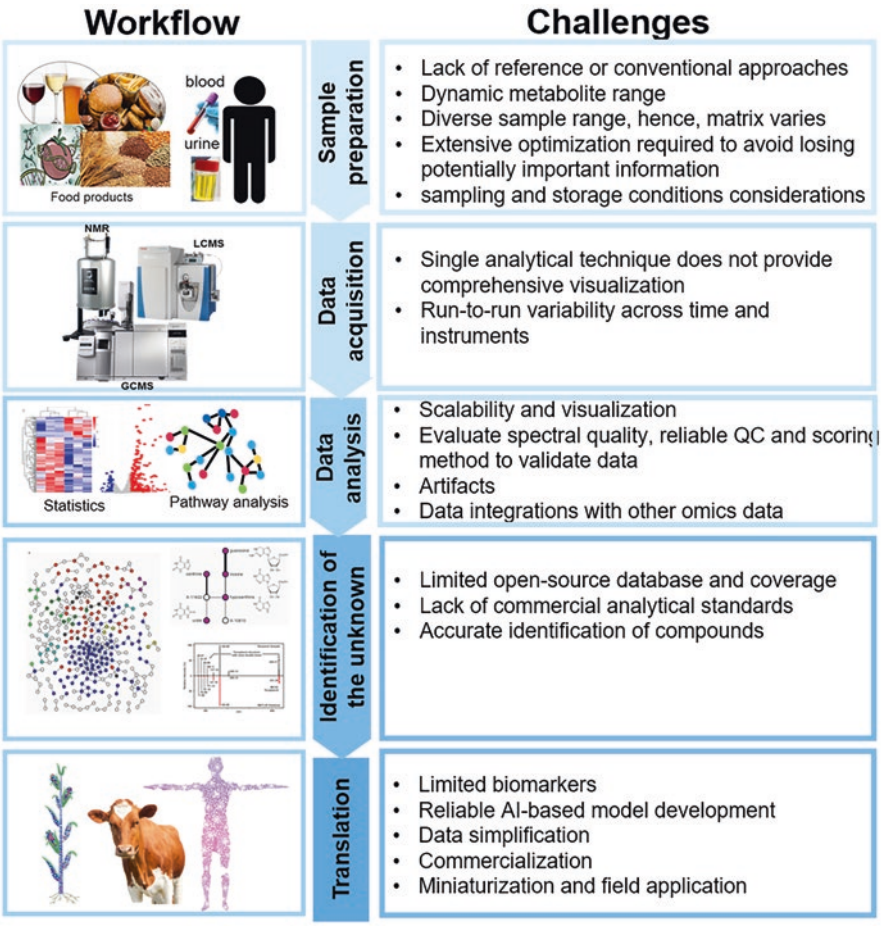

Fig. 5.6 Food metabolomics workflow and challanges

dietary polyphenols and their known human metabolites in over 400 foods (Rothwell et al. 2012). A similar ongoing project is PhytoHUB that will contain a comprehensive inventory of dietary phytochemicals and their human metabolites and structures either obtained from previous publications or predicted *in silico* (http://phytohub.eu/).

5.3.1 Soil Quality

Soil quality is one of the most critical factors determining plant health. The typical soil quality indicators are organic matter content, salinity, tilth, compaction, available nutrients, rooting depth, and microbial populations. Untargeted soil

Table 5.1 List of food metabolomics databases

#	Food metabolomics database	Information included	Public data
1	FoodData Central	Foods, compounds	Yes
2	FOODB	Foods, compounds	Yes
3	Exposome-Explorer	Foods, compounds, diseases	Yes
4	FoodComEx	Food compounds	Yes
5	PhytoHub	Food compounds	Yes
6	Phenol-Explorer	Polyphenol content in foods	
7	Human Metabolome Database (HMDB)	Small molecule metabolites	Yes
8	Chemical Entities of Biological Interest (ChEBI)	Small molecule metabolites	Yes
9	Dictionary of Food Compounds	Small molecule metabolites	No
10	KNApSAcK Core System	Plant metabolites	Yes
11	Dr. Duke's Phytochemical and Ethnobotanical Databases	Plant metabolites	Yes

metabolomics approaches are used to analyze extractable organic matter using LCMS (Swenson et al. 2015) and NMR (Johns et al. 2017) or discriminate the suppressive and non-suppressive soils for disease resistance (Rochfort et al. 2015). Soil metabolomics is also employed to assess the microbial (Boiteau et al. 2019; Rai et al. 2020) and plant metabolites (Petriacq et al. 2017; van Dam and Bouwmeester 2016) in soil.

Rai et al. has recently developed a high throughput method for the extraction and quantification of siderophores, highly specialized iron-chelating secondary metabolite, in the soil (Rai et al. 2020). Siderophores have ecological significance and find application as a biomarker for soil quality, the remedy of polluted sites, and improving nutrient metal uptake by crops or other plants. The detection and quantification of soil metabolites are challenging due to the complexity of soil matrix and metabolites' structural diversity. The workflow has the potentials to identify the soil biomarkers of plant health, as both plants and microbes secrete siderophores in response to iron limitation or cataloging soil-borne plant pathogens, a prerequisite for food security. On the other hand, identifying siderophores from beneficial rhizosphere bacteria can protect plants from pathogens and their virulence factors and keep them healthy. A yet another potential is discovering novel siderophores in the soil, which is obscure due to our inability to culture microbes in laboratory conditions. Microbes' critical need to sequester essential iron provides an achilles heel for new antibiotic development by utilizing the siderophore-based molecular recognition covalently attached to antimicrobial peptides. The unique metabolites of the soil-borne pathogens are used as biomarkers for plant diseases such as macrocarpa for *Rhizoctonia solani*-suppressive soil (Hayden et al. 2019) or siderophore of

beneficial bacteria that promotes or inhibits *Ralstonia solanacearum* infections (Pollak and Cordero 2020). Metabolomics is also employed to investigate the impact of soil microbial population on the leaf metabolome and herbivore feeding behavior (Badri et al. 2013).

5.3.2 Food Resources

Environmental factors such as geographical origins, application of chemicals (fertilizers, pesticides, industrial wastes), stress (abiotic and biotic), and genetic factors, including genetically modified (GM) crops in agriculture, significantly influence food resource production (Kim et al. 2016). Advancements in the analytical tools and ease to analyze a wide variety of plant samples ranging with varying culture conditions, geographic locations, growing seasons, exposure to stress, etc., could lead to the assessment of food resource quality changes due to environmental and genetic factors.

5.3.2.1 Metabolomics to Screen Geographic Origin

Geographical origins of food resources affect the quality and the prices, and hence is becoming an important issue for consumers and producers due to increasing international trade. It is almost impossible to determine the geographical origins of food resources by their phenotypes. Metabolomics is thus employed as a tool to assess the legitimacy and source of specific food resources such as hazelnuts (Klockmann et al. 2017), tobacco (Zhao et al. 2015), coffee (Arana et al. 2015), green tea (Lee et al. 2015), Spanish Extra Virgin Olive Oils (Gil-Solsona et al. 2016), grape (Teixeira et al. 2014), wine (Amargianitaki and Spyros 2017), milk and dairy products (Brescia 2005; Renou et al. 2004), honey (Zhou et al. 2018b), fish, and seafood (Cubero-Leon et al. 2014), beef (Man et al. 2021) since the metabolite profiles differ depending on their geographical origins. NMR fingerprinting is used to differentiate coffee beans based on country of origin (Arana et al. 2015). Zhao et al. correlated metabolite changes of carbon and nitrogen pools in tobacco plants from two different locations due to climatic factors such as rainfall and temperature (Arana et al. 2015).

5.3.2.2 Metabolomics for Organic Food Authentication

There is an increasing demand for **organic products**, and hence organic food items are expensive. The lack of reliable chemical markers to discriminate between organic and conventional products makes them an attractive target for fraudulent malpractices. Metabolomics could solve this problem by precisely detecting and quantifying the chemicals like pesticides or fertilizers in food products. The ability

of metabolomics to detect unknown targets is particularly beneficial to identify/detect new adulterants. A non-targeted metabolomics approach identifies food markers that discriminate between organic and conventional tomato crops (Martínez Bueno et al. 2018). Also, the presence of pesticides is determined in the amphibian liver (Van Meter et al. 2018), urine samples of healthy individuals (Reisdorph et al. 2020), and pregnant women (Sem et al. 2013). Growers sometimes apply growth promoter substances like testosterone, progesterone, auxin, and gibberellins beyond permissible limits to improve food resources quality and production yield. Metabolomics has recently been introduced as a new tool to detect illegal and excessive uses of growth promoters (Rodriguez-Celma et al. 2011; Šimura et al. 2018; Stephany 2010).

Organic agricultural systems rely on non-conventional soil fertilization techniques like the application of organic manures, biological pest controls, and multi-annual crop rotation, including legumes and other green manure crops. In contrast, chemical plant protection products, including pesticides, are applied to protect plants in conventional agriculture. Organic production systems increase the susceptibility of the plants to pathogens, hence resulting in the accumulation of inducible protective secondary metabolites such as phenolics; chlorogenic acid (Malik et al. 2009; Novotná et al. 2012; Young et al. 2005), and flavonoids (Mitchell et al. 2007). Metabolomics studies distinguish conventionally and organically grown produces (Martínez Bueno et al. 2018; Novotná et al. 2012; Vallverdu-Queralt et al. 2011). Factors such as plant response, rhizosphere microbiome are accountable (Bradi et al. 2013).

5.3.2.3 Metabolomics to Screen Genetically Modified (GM) Crops

Genetically modified (GM) food crops are resistant to diseases, pests, and unfavorable environmental conditions, produce high-quality foods with less effort and lower costs; however, GM food resources continue to be a controversial issue due to their safety and relation to human health and the environment (Simo et al. 2014). Organization for Economic Co-operation and Development (OECD), comprising 37 member countries, is established to globally deal with GM food resources safety issues (Kearns et al. 2021). OECD and European Food Safety Authority (EFSA) has developed guidance and regulations for GM crops to evaluate their safety and nutritional value (Bedair and Glenn 2020). Metabolomics provides comprehensive information about GM food composition compared to their corresponding non-GM counterparts (Simo et al. 2014). Metabolomics is effectively employed to assess the safety of widely consumed GM maize by comparing plasma metabolome and fecal microbiota in GM maize and non-GM near-isogenic maize-fed rats (Mesnage et al. 2019). Metabolomics is also employed to evaluate and assess GM food resources such as soybean (Garcia-Villalba et al. 2008; Inaba et al. 2007), rice (Jiao et al. 2010; Zhou et al. 2009), maize (Frank et al. 2012; Piccioni et al. 2009), wheat (Baker et al. 2006), tomato (Kusano et al. 2011b; Noteborn et al. 2000), potato (Catchpole et al. 2005), poplar (Srivastava et al. 2013), carrot (Cubero-Leon et al.

2018), and barley (Kogel et al. 2010). A recent study of GM rice with *Cr1C* gene transformation showed no significant difference in metabolic profile than the parent line (Chang et al. 2021). Another study reported differential metabolite profiles between wild-type and *cryIAc* and *sck* genes; for improving insect resistance in GM rice (Zhou et al. 2009). Targeted quantitative metabolomics could provide additional information for safety and nutritional assessment for GM crops with traits known to modify metabolic pathways. Integrating metabolomics with other omics data provides more comprehensive knowledge about risk assessment of GM crops (Kok et al. 2019). Metabolomics application for the safety assessment of GM crops, which is ever-growing and introduced to the global market, provides relevant information regarding the associated metabolite alteration. The challenges faced in such metabolomics applications include chemical complexity, identification of unknown metabolites, matrix effect as each plant is different, and dynamic concentration range. One key hurdle in using data from omics studies with GM crops, including metabolomics, is the difficulty to assess whether there is any impact on safety in the observed differences amongst the 1000s of signals characterized by the untargeted profiling method(s) (Bedair and Glenn 2020). Application of metabolomics to screen GM crops is infancy, and to get a comprehensive understanding of the detected metabolite changes in a biological context, big-data generated needs to be analyzed together with other 'omics' data such as proteomics and transcriptomics as proposed by the new Foodomics strategy (Ibanez et al. 2012). The development of advanced tools and databases is essential for metabolomics studies.

5.3.2.4 Metabolomics to Elucidate Environmental Stress Feedback in Food Resources

Environmental stress (biotic/abiotic) is a major limiting factor of agriculture production, affecting both yields and nutritional content. The early detection of stress symptoms could help reduce loss. Plant metabolomics has emerged as the most promising tool to decipher the metabolic changes caused by (a) climatic and seasonal variations, (b) biotic factors including pathogens and beneficial/symbiotic associations (Alseekh and Fernie 2018) for high-quality food resource production since the last decade.

Abiotic stress is responsible for global crop yield reduction ranging from 50% to 70% (Boyer 1982). Climate change and population growth have worsened the situation (Raza et al. 2019). Understanding plants' responses to such stressors to determine methods for improving crops quantitatively and qualitatively is inevitable. Abiotic stress in plants leads to the synthesis of phytohormones to impart stress resistance (Han et al. 2012; Rai et al. 2016); the oxidative stress disturbs the stomatal conductance and activates several signaling mechanisms and the dysregulated gene expression profiles (Rai et al. 2016). In particular, all essential plants' mechanisms from germination to maturity are severely affected by abiotic stresses. The major abiotic plant health stressors include drought, salinity, temperature extremes,

waterlogging, heavy metal, and chilling. Metabolomics has been applied for probing unique metabolites that regulate the abiotic stress tolerance mechanism in crops with two prime objectives: (i) identification of biomarkers for abiotic stress and (ii) investigate metabolic variations under abiotic stresses to detect different metabolites that permit restoration of plant homeostasis and normalize metabolic modifications (Arbona et al. 2013). **Water deficit (drought)** caused by global climate changes seriously endangers plant survival and crop productivity (Lesk et al. 2016). Metabolic profiling of drought-stressed wheat (Michaletti et al. 2018), barley (Chmielewska et al. 2016), rice (Lawas et al. 2019), and soybean (Das et al. 2017) are carried out to elucidate vital metabolites/biomarkers for drought tolerance. Soil **Salinity** is rapidly increasing, and about 20% of irrigated land is salt-affected. Salinity stress causes >20% losses in crop plants (Food and Agriculture Organization 2015). The GC-MS-based metabolic profiling of two salt-sensitive (Sujala and MTU 7029) and tolerant varieties (Bhutnath, and Nonabokra) of indica rice showed accumulation of two signaling molecules, serotonin, and gentisic acid, which may serve as a biomarker to produced salt-tolerant rice varieties (Gupta and De 2017). Metabolomics is employed to study metabolic remodeling due to salinity stress in several other crop plants like tomato (Rouphael et al. 2018), millet (Pan et al. 2020), strawberry (Antunes et al. 2019). **Temperature stress,** including heat (Abdelrahman et al. 2020; Escandon et al. 2018; Raza 2020) or cold (Furtauer et al. 2019; Xu et al. 2020), disturbs the homeostasis and physiological mechanisms. Metabolomics analysis of temperature-stressed wheat (Qi et al. 2017; Thomason et al. 2018), tomato (Almeida et al. 2020; Paupiere et al. 2017; Zhang et al. 2019), and maize (Obata et al. 2015; Sun et al. 2016; Urrutia et al. 2021) are studied to identify the effect of temperature stress. **Heavy metal** stress has become a significant concern on various terrestrial ecosystems due to extensive industrialization (Guerrero et al. 2019; Shahid et al. 2015). Suboptimal concentrations of trace metals such as Zn, Cu, Mo, Mn, Co, Ni, As, Pb, Cd, Hg, Cr, and Al reduce crop metabolism, growth, and productivity (Tiwari and Lata 2018). Metabolomics analysis of high Zn and Cu treated beans (Jahangir et al. 2008), Zn-deficient tea plants (Zhang et al. 2017), heavy metal stressed (Cu, Fe, and Mn) Brassica rapa (Jahangir et al. 2008), Cr-toxicated sunflower (Gonzalez Ibarra et al. 2017) and Fe-toxicated rice (Turhadi et al. 2019) are performed. A better understanding of the **nutrient-limitation**, including macro- and micro-nutrients, would enhance the food/fodder nutritional contents. Metabolic changes due to macro-nutrient: nitrogen (Kusano et al. 2011a; Rai et al. 2017), phosphorus (Jones et al. 2018; Vance et al. 2003), and potassium (Zeng et al. 2018), deficiency has been studied in plants/microalgae using HPLC and enzymatic activities (Scheible et al. 2004; Tschoep et al. 2009), FT-ICR-MS (Hirai et al. 2004), LC-MS (Peng et al. 2008), GC-MS (Heyneke et al. 2017; Urbanczyk-Wochniak and Fernie 2005), CE-MS (Takahara et al. 2010; Takahashi et al. 2009), 1H-NMR (Broyart et al. 2010).

Plant-microbe (pathogenic/beneficial) interactions (**Biotic factors**) trigger a plethora of primary and secondary metabolites changes, which could be easily detected by metabolomics (Allwood et al. 2008; Castro-Moretti et al. 2020). A wide range of phytopathogens, including fungi, bacteria, viruses, viroids, mollicutes,

parasitic higher plants, and protozoa, are known to cause plant disease. Metabolomics is an emerging tool to study plant-pathogen interactions (Castro-Moretti et al. 2020). An integrated metabolo-proteomic approach showed induced phenolic acid and phenylpropanoids in *Fusarium graminearum* infected wheat (Gunnaiah et al. 2012). NMR analysis indicated an increased accumulation of disease-resistant biomarkers (Sarrocco et al. 2016). Metabolomic analysis of the susceptible and resistant wheat cultivars infected with the fungal pathogen *Zymoseptoria tritici* showed that immune and defense-related metabolites in resistant and susceptible wheat cultivars using FT-ICR-MS (Seybold et al. 2020). Likewise, the metabolomics responses in wheat against viral infection; wheat streak mosaic virus (Farahbakhsh et al. 2019), rice against fungal infection; *Rhizoctonia solani* (Suharti et al. 2016), *Magnaporthe grisea* (Jones et al. 2010), insect attack; gall midge (Agarrwal et al. 2014), bacterial infection; *Xanthomonas oryzae* pv. oryzae (Sana et al. 2010), maize against fungal infection; *Fusarium graminearum* (Zhou et al. 2018a), southern corn leaf blight (Vasmatkar et al. 2019), and insect attack; *Ostrinia furnacalis* (Guo et al. 2019) are studied. Adverse environmental conditions, including temperature, soil fertility, light, water deficit, give an edge to the pathogens and increase disease severity. However, we have very little knowledge about these tripartite interactions, suggesting future investigations towards understanding the multi-dimensional nature of plant-pathogen interactions in changing climate conditions (Velásquez et al. 2018). Weeds are yet another problem that impacts crop productivity at multiple levels, such as competing for nutrients, reducing crop yields and nutritional content, interfering with harvest efficiency, and recurrence. Metabolomics analysis of the canola plant extracts detected allelopathic metabolites (3,5,6,7,8-pentahydroxy flavones, p-hydroxybenzoic acid, and sinapyl alcohol) that inhibit the rye root and shoot development (Asaduzzaman et al. 2015). Similarly, weed-suppressing metabolites in wheat and legume are investigated (Latif et al. 2019).

5.3.2.5 Metabolomics in Livestock Research

Traditional livestock analysis, such as feed consumption, is time-consuming, expensive, and requires specific equipment (Karisa et al. 2014). Reproductive trait measurements need animals to reach the maturity stage, while carcass trait evaluation requires animal slaughter which otherwise could have been used for breeding. Metabolomics has emerged as an efficient, cost-effective, non-invasive way to detect animal traits for livestock research and industry. Livestock metabolomics is instrumental in animal breeding farm trials for efficient and quicker quantitative phenotyping (Karisa et al. 2014; te Pas et al. 2017) and is primarily applied for biomarker identification for weight gain, milk quality (D'Auria et al. 2013), health (LeBlanc et al. 2005), fertility (Chapinal et al. 2012), etc. The metabolomic signatures associated with feed efficiency in beef cattle (Novais et al. 2019), dairy cow (Saleem et al. 2012) is studied. Also, metabolomics is employed to analyze antimicrobial resistance in livestock pathogens like *E. coli* (Campos and Zampieri 2019; Lin et al. 2019). Livestock metabolomics experimental design should take diet,

diurnal variations, sex, and sampling time into consideration to reduce variability. Shortage of data resources makes data interpretation a challenge in livestock metabolomics Goldansaz et al. created a livestock metabolome database of more than 1000 metabolites detected in livestock metabolomic studies on cattle, sheep, goats, horses, and pigs (Goldansaz et al. 2017). Bovine Metabolome Database is a recent collection of more than 50,000 metabolites focusing on animal health which describes a healthy range of metabolites in bovine biofluids and tissues (Foroutan et al. 2020).

5.3.3 Food Processing

Our modern lifestyle and the ever-growing global population have caused increased food processing industry demands. Food processing can be defined as the physical and/or chemical manipulation of raw food to enhance nutritional and sensory quality and sustainability. Some of the food processes where metabolomics is applied include cheese (Afshari et al. 2020), tomato purees (Capanoglu et al. 2008), tinned vegetables, biscuits (Diez-Simon et al. 2019), alcoholic beverages (Álvarez-Fernández et al. 2015; Ichikawa et al. 2019), yogurt (Settachaimongkon et al. 2015) and milk (Rocchetti et al. 2020). Metabolomics finds its role in the **food processing** industry ranging from food preparation, packaging, and storage. For instance, food preparation processes like pasteurization, fermentation, etc., could affect the food's nutritional and sensory quality either beneficially (improved digestibility, nutrients bioavailability, foodborne pathogens/toxins inactivation) or detrimentally (vitamins and nutrient loss, toxic compounds formation, conferring adverse effects on flavor, aroma, texture or color), monitored by metabolomics. Another exciting application of metabolomics is identifying the chemical species that contribute to flavor, texture, taste, and color quality, which could be used to develop meat using plants, considering the growing demand for plant-based foods (https://www.impossiblefoods.com/food). Metabolomics is applied in **food safety** for rapid and reliable monitoring of food contaminants (i) pesticides and other chemical residues like furans, dioxins, dioxin-like polychlorinated biphenyls (PCBs), non-dioxin-like PCBs (Tengstrand et al. 2012; Zainudin et al. 2015), (ii) foodborne pathogens like *Salmonella* sp., *E. coli* (Cevallos-Cevallos et al. 2011) and *Listeria* sp. (Jadhav et al. 2015), with short-term and long-term health risks (Pinu 2016). Predicting the end of shelf life before apparent spoilage (expiry date or best before) and determining effects of food processing on the shelf life is another application of metabolomics. For instance, metabolic profiling showed a correlation between diphenylamine oxidation treatment and extended shelf-life during storage in apples (Leisso et al. 2013). Likewise, the shelf life of meat at various storage temperatures was studied (Argyri et al. 2015). A comprehensive understanding of food metabolite composition during processing and storage will improve preservation methods. Metabolomic assessment of **food packaging** types is performed to detect contaminants and spoilage in packaging materials (Makkliang et al. 2015). Also, metabolomics could

expand our understanding of the biodegradable materials used for packaging (Kleeberg et al. 1998) and microbial and plant strain improvement for efficient production of eco-friendly packaging materials.

5.3.4 Human Nutrition

Diet/nutrition is linked to metabolic disorders, including obesity, diabetes, cardiovascular disease, and aging (Shlisky et al. 2017). The traditional way to evaluate beneficial or detrimental effects of foods based on volunteers filling the questionnaires has several limitations: misreporting, bias and measurement error, high cost and time consumption, and unreliability for populations with cognitive impairment (Fallaize et al. 2014). Metabolomics is a powerful tool to overcome such limitations, and hence dietary biomarkers are emerging as an objective and accurate measure of dietary intake and nutrient status (O'Gorman et al. 2013). Novel metabolic signatures are associated with juice and fruits (Liu et al. 2015), grain, fish (Hanhineva et al. 2015), wine (Urpi-Sarda et al. 2015), and diet patterns (western vs. prudent) (Bouchard-Mercier et al. 2013). Also, metabolomics is used to monitor diet-related metabolic diseases (Sebedio 2017). LC/MS-based metabolic profiling suggested green tea mediated stimulation of hepatic lipid metabolism associated with obesity prevention (Lee et al. 2015). Likewise, the anti-hyperlipidemia effect of curcumin was demonstrated using NMR and MS-based metabolomics (Li et al. 2015). A yet another hot area of metabolomics research is the human gut-microbiota study. Recent studies have suggested that the gut microbiome secretes metabolites that impact human health (Dore and Blottiere 2015). Moreover, the gut microbiome varies between individuals and is greatly affected by diet. Interestingly, diet changes can manipulate the gut microbiome, currently being studied for use as a potential therapy (Shoaie et al. 2015). Selected food metabolomics studies are listed in Table 5.2.

5.4 Challenges in Food Metabolomics

Despite its enormous potential, there are several dark areas of food metabolomics: (i) lack of optimized workflow for sample processing, normalization, and data analysis, (ii) run-to-run variability, (iii) substantial matrix effects, (iv) limited spectral library coverage, (iv) availability of open-source data analysis tools and databases, and (v) lack of comprehensive guidelines for biomarker prediction and validation for food resources. To overcome the challenges of experimental design, power calculation should be performed to determine the sample size required for statistically significant results and reduce variability, particularly for field samples. Cultivated varieties, geographical location, and fertilization schemes should be taken into consideration. Quality control samples should be included to determine run-to-run

Table 5.2 Comprehensive list of food metabolomics study

Food metabolomics	Treatments	Tissue	Sample	Primary/Secondary metabolites	Analytical platform	Reference
Plant food resources	Genetic lines	Fruit	Apple	Primary and secondary	GC-MS	Cuthbertson et al. (2012)
		Fruit	Grape	Primary and secondary	1H NMR	Fortes et al. (2011)
		Fruit	Melon	Primary and secondary	1H NMR, FIE-MS, SPMEGC-MS, GC-MS	Allwood et al. (2014) and Bernillon et al. (2012)
		Fruit	Tomato	Primary and secondary	GC-MS, LC-MS	Gomez-Romero et al. (2010) and Schauer et al. (2006)
		Fruit	Pepper	Primary and secondary	LC-MS, GC-MS	Wahyuni et al. (2013)
		Flower	Broccoli	Primary and secondary	LC-MS	Sun et al. (2015)
		Leaf and fruit	Tomato	Primary	GC-MS	Roessner-Tunali et al. (2003)
		Grain	Rice	Primary and secondary	1H NMR, GC-MS	Calingacion et al. (2011) and Lou et al. (2011)
		Tuber	Potato	Primary	GC-MS	Roessner et al. (2000)
	Natural accessions	Tuber	Potato	Primary	GC–MS	Carreno-Quintero et al. (2012)
		Grain	Maize	Secondary	LC-MS	Lipka et al. (2013) and Owens et al. (2014)
		Vegetable	Tomato	Metabolome	GC–MS	Sauvage et al. (2014)
		Kernel	Maize	Primary and secondary	LC–MS	Shen et al. (2013) and Wen et al. (2014)
	Doubled haploid lines	Flag leaf	Wheat	Metabolites	LC–ESI–MS, GC-MS	Hill et al. (2013, 2015)

(continued)

Table 5.2 (continued)

Food metabolomics	Treatments		Tissue	Sample	Primary/Secondary metabolites	Analytical platform	Reference
	Chromosomal segment substitution lines		Seed	Rice	Primary	LC-Q-TOF-MS	Matsuda et al. (2012)
	Introgression lines		Vegetable	Tomato	Primary and secondary	GC–MS, LC-MS	Alseekh et al. (2015), Perez-Fons et al. (2014), Schauer et al. (2008), Schauer et al. (2006), and Toubiana et al. (2012)
	Abiotic stress	Drought	Leaf	Rice	Primary and secondary	GC/EI-TOF-MS	Degenkolbe et al. (2013), Do et al. (2013), and Ma et al. (2016)
			Leaf	Soybean	Primary	1H NMR	Silvente et al. (2012)
			Leaf	Sorghum	Primary and secondary	FT-IR and GC/MS	Ogbaga et al. (2016)
			Leaf	Maize	Primary	GC/MS	Obata et al. (2015)
			Multiple tissues	Maize	Primary	GC-TOF-MS	Witt et al. (2012)
			Immature kernels	Maize	Primary	RP/UPLC -MS/MS	Yang et al. (2018)
			Root, shoot, leaf and flag leaf	Wheat	Primary and secondary	GC-MS	Guo et al. (2018), Kang et al. (2019), and Yadav et al. (2019)

		Salt	Leaf and root	Rice	Primary and secondary	GC/MS, NMR	Chang et al. (2019), Gupta and De (2017), and Ma et al. (2018)
			Seedling	Rice	Primary and secondary	GC/MS	Gayen et al. (2019)
			Leaf	Wheat	Primary and secondary	GC/MS	Che-Othman et al. (2020) and Guo et al. (2015)
			Leaf	Maize	Primary and secondary	GC-MS	Zorb et al. (2013)
			Roots	Barley	Primary and secondary	GC-MS	Shelden et al. (2016)
			Roots and shoots	Wheat	Primary and secondary	HPLC	Borrelli et al. (2018)
			Terminal leaflet	Tomato	Primary and secondary	UHPLC-ESI/QTOF-MS	Rouphael et al. (2018)
		Heat	Filling grains	Wheat	Primary and secondary	LC-MS/MS HPLC	Wang et al. (2018)
			Flag leaves	Wheat	Primary and secondary	LC-HRMS	Thomason et al. (2018)
		Waterlogging	Root and leaf	Soybean	Primary and secondary	CE/MS, NMR	Coutinho et al. (2018) and Komatsu et al. (2014)
			Leaf	Rice	Primary and secondary	GC/MS, NMR	Barding Jr. et al. (2013) and Locke et al. (2018)
			Shoot	Wheat	Primary and secondary	GC/MS, LC/MS	Herzog et al. (2018)

(continued)

Table 5.2 (continued)

Food metabolomics	Treatments		Tissue	Sample	Primary/Secondary metabolites	Analytical platform	Reference
	Biotic stress	*Zymoseptoria tritici*, *Fusarium graminearum*, Wheat streak mosaic virus, *Triticum turgidum*	Leaf	Wheat	Primary and secondary	FT-ICR-MS, NMR, LC-MS	Cuperlovic-Culf et al. (2019), Farahbakhsh et al. (2019), Seybold et al. (2020), and Shavit et al. (2018)
		Fusarium graminearum	Rachis and spikelet	Wheat	Primary and secondary	NMR, LC-LTQ-Orbitrap	Cuperlovic-Culf et al. (2016) and Gunnaiah et al. (2012)
		Orseolia royzae, *Xanthomonas oryzae* pv. Oryzae, *Magnaporthe grisea*, *Rhizoctonia solani*, *Chilo suppressalis*, Nilaparvata lugens	Leaf	Rice	Primary and secondary	GC/MS, LC/MS, NMR, CE/TOF-MS	Agarrwal et al. (2014), Jones et al. (2010), Liu et al. (2016), Peng et al. (2016), Sana et al. (2010), and Suharti et al. (2016)
		Lolium perenne	Root and shoot	Rice	Primary and secondary	LC-QTOF-MS	Asaduzzaman et al. (2014)
		Bipolaris maydis, *Ostrinia furnacalis*	Leaf	Maize	Primary and secondary	FT-IR, NMR, LC-MS	Guo et al. (2019) and Vasmatkar et al. (2019)
		Fusarium graminearum	Roots	Maize	Primary and secondary	LC/MS	Zhou et al. (2019)
		Pseudomonas syringae pv	Leaf	Tomato	Primary and secondary	NMR and LC/MS	Lopez-Gresa et al. (2010)
	Organic vs. conventional		Grain	Wheat	Primary	GC-MS	Zorb et al. (2006)

Livestocks	Stored for different periods	Beef	Beef	Primary	1H NMR	Graham et al. (2010) and Savorani et al. (2010)
	Genetic lines	Fish	Gilthead sea bream	Primary	1H NMR	Savorani et al. (2010)
		Liver and white muscle	Salmon	Primary	1H NMR	Wagner et al. (2014)
		Meat	Pork	Primary	1H NMR	Straadt et al. (2014)
	Three different aquaculture systems	Meat	Gilthead sea bream	Primary	1H NMR	Savorani et al. (2010)
Processed food	Genetic lines	Oil	Olive oil	Secondary	LC-MS	Sanchez de Medina et al. (2014)
		Orange juice	Mandarin oranges	Primary	1H NMR	Zhang et al. (2012)
	Changes in food composition during postharvest handling, processing, and storage	Wine	Grapes	Primary and secondary	1H NMR, GC-MS	Pinu et al. (2013)
			Rice (Japanese sake)	Primary	CE-MS, LC-MS	Sugimoto et al. (2012)
		Grains	Barley	Primary and secondary	GC-MS	Frank et al. (2011)
		Edible part	Broccoli, tomato, and carrot	Primary and secondary	1H NMR, LC-MS, LC-MRM, GC-MS	Lopez-Sanchez et al. (2015)
		Tomato paste	Tomato	Primary and secondary	LC-MS	Capanoglu et al. (2008)
		Fruits	Peach	Primary and secondary	GC-MS	Lauxmann et al. (2014)
		Pasta	Semolina pasta	Primary and secondary	LC-MS, GC-MS	Beleggia et al. (2011)
		Soy sauce	Soybeans	Primary metabolites	1H NMR	Ko et al. (2009)

(continued)

Table 5.2 (continued)

Food metabolomics	Treatments		Tissue	Sample	Primary/Secondary metabolites	Analytical platform	Reference
	Storage temperature		Beer	Beer	Primary and secondary	LC-MS	Heuberger et al. (2012)
	Various Bacillus strains		Fermented soybean paste	Soybean	Primary	GC-MS	Baek et al. (2010)
	Foods based on characteristic metabolite profiles		Cheese	Mozzarella cheese	Primary	1H NMR	Mazzei and Piccolo (2012)
			Wine	Wine	Primary	1H NMR	Lopez-Rituerto et al. (2012)
	Organic vs. conventional		Puree	Ketchup	Primary and secondary	LC-MS	Vallverdu-Queralt et al. (2011)
Human nutrition	Uptake as food	Milk and meat	Serum and urine	Human	Primary and secondary	LCMS	Bertram et al. (2007)
		Dietary modulation	Urine	Human	Primary and secondary	1H NMR	Stella et al. (2006)
		Well-cooked chicken	Urine	Human	Primary and secondary	LCMS/MS	Kulp et al. (2004)
		Gelatin	Serum	Human	Primary and secondary	LCMS/MS	Ichikawa et al. (2010)
		Citrus	Urine	Human	Primary and secondary	1H NMR	Heinzmann et al. (2010)

variability; any data showing coefficient of variance higher than 20% should be discarded. The lack of external validation sets using samples, which are not part of the statistical models built for prediction, is a significant limitation in most studies published (Cubero-Leon et al. 2018). Matrix effects could to either eliminated by reducing the sample complexity or spiking the standards in the matrix. Absolute metabolite quantification workflows should be improved using a combination of different analytical tools. So far, targeted approaches are used for metabolite quantification, efforts for developing untargeted quantitative metabolomics methods are needed for efficient biomarker discovery. Further regulatory guidelines for biomarker discovery should be put in place for robust experimental design, data acquisition, validation, and translation. Extensive research is required for the instrument miniaturization, cost-effectiveness, accessibility, and ease-of-handing for the fieldable application of metabolomics in agriculture farms. This could be achieved by corroboration between farm workers, researchers, and engineers. Automated, user-friendly, and open-source metabolomics data analysis platforms should be developed for robust data interpretation.

5.5 Conclusion

With ever-increasing food demand, climate change, and the advent of food metabolomics in the past decade, the horizon of metabolomics application has increased from food resource production to food processing and human nutrition. Because of increased efficiency, cost-effectiveness, and accuracy, applications of food metabolomics are rapidly expanding, as discussed extensively in this chapter. Untargeted metabolomics is especially useful in detecting unknown adulterants or advancing food metabolomics research (Cubero-Leon et al. 2014). Food databases are handy in elucidating the unknown/novel metabolites (Table 5.1). Plant metabolite repertoire has a wide dynamic range and diverse chemical composition. With the current set-up, only ~10% of the metabolite are identified; hence there is enormous scope to explore the unknowns. Integrating the metabolomics information with other-omics (genomics, transcriptomics, and proteomics) and non-omics studies (physiological data), combined with reliable and broad-spectrum food metabolome databases and artificial intelligence technologies, could help identify and elucidate many ‘unknowns’ and monitor in real-time to predict dynamics and quality control for accelerating, automating and progressing production processing.

5.6 Perspective

Apart from the applications of food metabolomics discussed in this chapter, metabolomics could be employed in personalized nutrition, crop improvement, metabolic fingerprinting in livestock and plants. Metabolomics investigations have generated

a plethora of information that will allow food manufacturers to develop nutritional and sensorial rich food. Metabolomics-assisted crop improvement could lead to high-yielding, stress-tolerant germplasm and create climate-smart crop varieties. However, deciphering a specific metabolite's function (metabolite-phenotype) and decoding the structure of metabolic networks remains a major hurdle in the third decade of plant metabolomics. Identifying biomarkers related to plant biotic/abiotic stress, genetically modified organisms (GMOs), organic vs. conventional produce, and human nutrition using metabolomics and its translation in the agricultural fields, food industry, and clinics could be a future application. Also, metabolomics allows the determination of nutrient enhancement or reduction due to food processing via detection chemical alteration, which could significantly alter human health. Fingerprinting metabolic phenotypes of livestock and plants in response to defined feeding/fertilizing patterns and compositions is another emerging application. Finally, the food waste generated as a by-product of agriculture, the food processing industry, or household could be used to extract high-value bioactive compounds and nutraceuticals. In the years to come, an innovative sequential approach could simultaneously use food resources for food processing and simultaneously extract specific bioactive compounds and nutraceuticals to be used as functional foods.

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Chapter 6
Phytobioactive Compounds on Ovarian Functions in Mammals

V. Girish Kumar, B. Sampath Kumar, Poonam Kumari Singh, and S. Nandi

Abstract Carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organo-sulphur compounds are major bioactive plant compounds that can be used to improve the mammalian reproduction. These compounds are alternatives to and show less side effects than synthetic drugs.

Here we review the effects of phytobioactive compounds on mammalian ovarian functions, with focus on kaempeferol, quercetin, myricetin, curcumin, and resveratrol. These compounds target specific compounds in the ovarian cells, such as hormones, enzymes, and reactive oxygen species. Molecular and cellular interactions of phytobioactive compounds assists the plant therapist in developing a novel, cost-effective, commercially available plant-based medication with minimal side effects.

Keywords Phytobioactive compounds · Mammalian reproduction · Ovarian function

Abbreviations

Kaempferol	KAE
Quercetin	QUE
Myricetin	MYR

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Curcumin	CUR
Resveratrol	REV
ROS	Reactive oxygen species
SOD	Superoxide dismutase
MMP	Mitochondrial membrane potential
VEGF	Vascular epithelial growth factor
MAPK	Mitogen activated protein kinase
JNK	c-Jun N-Terminal kinase
NF-κB	Nuclear factor kappa B
SIRT1	Silent mating type information regulator 1
PI3K/Akt	phosphatidylinositol-3- kinase /protein kinase B
Nrf2-ARE	Nuclear erythroid 2-related factor-Antioxidant related element

6.1 Introduction

Medicinal plants are amazing natural elements that can be used to cure a variety of human and animal illnesses (Tran et al. 2020). Around 80% of the world's population currently relies on plants to treat essential diseases, most often by extracts or bioactive ingredients (WHO 2019). Similarly, bioactive substances from plants have been used as drugs to treat various reproductive disorders in mammals since decades (Atanasov et al. 2015). In reality, plant-derived bioactive compounds are the main therapeutic agents for a variety of sexual disorders in mammals (Zhao et al. 2015). Moreover, the phytobioactive compounds are potential substances that can interact with certain components of live tissue to trigger the various effects (Guaadaoui et al. 2014). Hence, plant-based medications and/or their bioactive compounds may be used as alternative therapies in the field of reproductive biology, either directly or with certain chemical changes (Chandra et al. 2017). In addition, it replaces the use of exogenous hormones or negative growth factors. However, if plants and/or plant-based medications are to be ingested as medication, a detailed analysis of cellular and molecular mechanisms would be needed, as it is a dose-dependent effect (Aju et al. 2017). As a result, the foregoing information is summarized here in order to gain a better understanding of the effects of such phytobioactive compounds on ovarian functions in mammals. This would also facilitate in the development of a potential folkloric phytochemical therapeutic agent with a broad spectrum of action as well as little side effects as compared to synthetic drugs.

6.2 Effective Bioactive Compounds on the Mammalian Ovarian Function

Phytochemicals present in medicinal plants are classified into **1. Carotenoids** (40 carbon units bonded together by alternating single and double bonds): alpha-carotene, beta-carotene, lycopene, etc. **2. Phenolics** (hydroxyl group attached to an aromatic ring): phenolic acids, flavonoids, stilbenes, coumarins, tannins. **3. Alkaloids**, **4. Nitrogen-containing** (carbon rings with the nitrogen atom inside) and **5. Organo-sulfur compounds** based on the biochemical structural difference (Liu 2004). Among the above, carotenoids (FSHR and LHR receptor induction), Phenolics (Regulation of Foxo3/signal pathways) and alkaloids (Effect on CNS) play a key role in augmenting the reproductive ability in mammals (Kushwaha et al. 2014). However, among the other bioactive substances, Kaempferol, Quercetin, Myricetin (flavanolic phenols), Curcumin (Polyphenol), and Resveratrol (stillbenic phenol) have been shown to have great outcomes on mammalian ovarian functions (Fig. 6.1). The bioactive compounds mentioned above can be found in the different plant sources listed in Fig. 6.1.

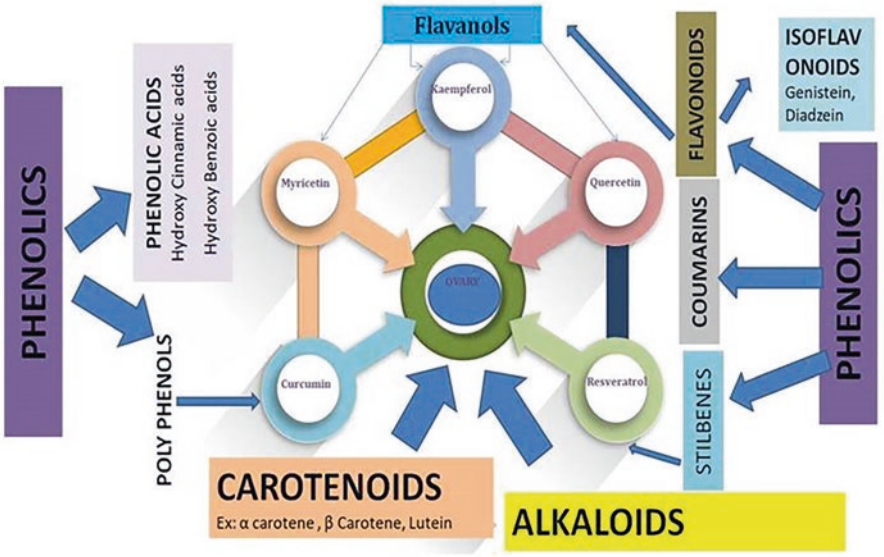

Fig. 6.1 Bioactive compounds on the mammalian ovarian function

6.2.1 Kaempferol

Kaempferol (KAE) is a flavonol that is polyphenolic (Liu 2004). This bioactive compound has a significant role in the development of viable ovarian follicles with active mitochondria (primary and secondary) (Yao et al. 2019). It also aids in the reduction of DNA fragmentation in ovine and porcine cultured follicles (Santos et al. 2019).

The source and availability of kaempferol is give in the Fig. 6.2. Kaempferitrin (Vishnu Prasad et al. 2009), astragalin (Wei et al. 2011), afzelin (Markham et al. 1992), kaempferol, 7-O-glucoside (Ibrahim et al. 2008), robininine (March et al. 2004), sophoraflavonoloside (Kim et al. 2012) and trifolin (Nowak and Wolbiś 2002) are the gycoside-shaped plants of this category of substance. The KAE, also known as kaemperol 3 or kaempferide, is a chemical product of bioactive plant metabolism (Curir et al. 2001). Naturally occurring KAE and/or secondary glycosidic metabolites of kaempferoid have a variety of biological actions and are therefore used as an agent (anticancer, antioxidant, antiglycine, estrogenic, and so on) in the treatment of a variety of disorders (Hung 2004).

6.2.1.1 Structure

Kaempferol (KAE) is a flavonol-type flavonoid chemically it is a 3,4′,5,7-tetrahydroxyflavone with a M.W. of 286.2 g/mol (Santos et al. 2019). Structurally KAE is a tetrahydroxyflavne in which the four hydroxyl groups are located at position 3,5,7 and 4′ (Fig. 6.2) (Calderon-Montano et al. 2011).

6.2.1.2 Molecular Targets

The addition of KAE to the culture media (follicle, oocyte, embryo) is having a dose-dependent action. For instance, at a concentration of 10 μM, it could able to enhance the primordial follicle activation, cell proliferation and oocyte meiotic resumption in the ovine cultured follicles (Santos et al. 2019). Similarly, at 0.1 μm concentration, KAE was effective in increasing the blastocyst number as well as its formation rate in the porcine cultured follicles (Zhao et al. 2019). Likewise, at 1 μM concentration also KAE was proven as effective to increase the follicle diameter and

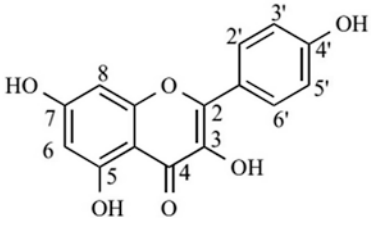

Fig. 6.2 Kaempferol molecular structure

oocyte growth in other mammals as well (Zhou et al. 2015). The above specific effect was executed by increasing the antioxidant enzyme expression levels (catalase, heme-oxygenase and GSH) (decreases lipid peroxidation) (Zhou et al. 2015).

Advancing to the molecular actions of KAE, it acts in multiple ways. One of the major mechanisms is PI3K/Akt (phosphatidylinositol-3- kinase/protein kinase B) pathway. KAE has effects on ovine preantral follicles by this pathway (*invitro* culture) (Santos et al. 2019). In addition, another KAE mechanism was to increase COX2 and SOX2 mRNA expression of the genes (the function of the embryonic development) with a substantial decrease in Caspase-3 expression levels. Furthermore, by reducing the oxidative stress KAE protects the oocytes during in vitro maturation. KAE executes this action through various mechanisms. One among them is by up-regulating the mitochondrial SIRT1 gene expression (Guo et al. 2015). Likewise, another was Nrf2-ARE (Nuclear erythroid 2-related factor-Antioxidant related element) up regulation (Saw et al. 2014) by augmenting the levels of p-p38, Nrf2, SOD, and catalase (Kim et al. 2012). Nevertheless, it is an undeniable fact that the continuous production of ROS and/or calcium overload can target the mitochondria and can create oxidative stress (Ott et al. 2007). KAE was effective in ameliorating the above deleterious effect either by enhancing the mitochondrial membrane potential (MMP) (porcine embryos) (Guo et al. 2015) and/or reducing the MC3T3-E1 intracellular Ca2+ concentrations (Choi et al. 2011).

Despite all the above effective mechanisms, an indelible action of KAE was also seen on aging oocytes. KAE could able to delays the oocyte aging and can improve the subsequent embryonic development cascade (Yao et al. 2019). This further executed in the aging oocytes either by reducing the apoptosis (decreasing ROS levels) and/or maintaining the sufficient MMP (Yao et al. 2019). Another reason attributed to the improvement of KAE treated aged oocytes could be an increase in the mRNA expression levels of *ITGA5, NANOG, Oct4* and *SIRT1* genes (transcriptional factors role in embryonic pluripotency) in the porcine (Zhang et al. 2016). Furthermore, few reports revealed a phytoestrogen activity of KAE, which is also dose-dependent (Kim and Choi 2013).

6.2.1.3 Effect on In-Vitro Folliculogenesis and Oocyte Culture

KAE helps in the maintenance of ovarian follicular survival, promotion of active mitochondria level as well as oocyte meiotic resumption (Santos et al. 2019). Likewise, KAE (10 μM concentration) promoted the primordial follicle activation and cell proliferation via the PI3K/Akt pathway in ovine preantral follicles cultured in vitro. At the same concentration, KAE also reduces the DNA fragmentation (Santos et al. 2019). Further, KAE also played a major role in the development of ovine ovarian secondary follicles (antrum formation) (Santos et al. 2019) and porcine preantral follicles (Tasaki et al. 2013) cultured in vitro by binding with estrogen receptors (ERs) (Kim and Choi 2013). The chemical resemblance to endogenous steroid hormones, such as estradiol, is responsible for this KAE action (Kim and Choi 2013). Further, this phytoestrogen activity of KAE also helps in consequently

achieving the follicular development as well as good quality of oocytes (Cadenas et al. 2017). Similarly, KAE also has proven as an effective bioactive compound in the porcine oocyte culture medium, where the addition of 0.1 μm concentration of KAE increases the rate of blastocyst formation compared to control oocytes (Zhao et al. 2019). Likewise, The KAE-treated porcine-oocyte blastocyst has demonstrated a substantial increase in COX2 and SOX2 mRNA expression levels (development related genes). While the Caspase-3 gene (apoptosis associated gene) was shown to show substantially lower levels of mRNA expression (Zhao et al. 2020).

The reality is that reactive oxygen species (ROS) exposure will increase in *in vitro* conditions, resulting in mitochondrial damage, reduced quality oocytes, and cell death (Succu et al. 2011). In the in vitro culture medium of ovine secondary follicles, KAE (1 μM concentration) can be added as an antioxidant to replace transferrin, Selenium and Ascorbic acid in vitro culture medium. In addition, KAE can trigger anti-oxidant properties, which in turn increase follicle Survival, follicular diameter as well as oocyte formation, by preventing lipid peroxidation (Lee et al. 2010) as well as by enhancing the anti-oxidant expression (catsalases, Hem-oxygenase and GSH). Likewise, KAE also improves the pathway (key mediator in oxidative stress) for the upregulation of cellular processes (homeostatics, oxidative stress and ageing), as well as the protection of the oocytes against oxidative stress during invitro maturation, with mitochondrial sirtuin (Sirt3) (Yang et al. 2019) and Nrf2-ARE (Nuclear erythroid, factor-antioxidant-associated element) increment (Saw et al. 2014).

6.2.2 Quercetin

Quercetin (QUE) is an ovarian modulatory flavonolic bioflavonoid. Mostly used in the regulation of ovarian functions (Ovarian folliculogenesis, oocyte maturation and ovulation) (Santini et al. 2009). Further, QUE acts on ovarian follicular cells and oocytes either by enhancing the mucification process, mitochondrial activity and/or by controlling the DNA fragmentation (Tarazona et al. 2006).

QUE is a beneficial plant bioflavonoid with M.W. of 302.23 g/mol (Chemical name) (Lakhanpal and Rai 2007). Characteristically QUE is yellow in color, soluble in alcohol and lipids, insoluble in cold water (David et al. 2016).

6.2.2.1 Structure

Structurally QUE is a 3,3′,4′,5,7- pentahydroxyflavone with diverse biological functions (anti-inflammatory, anti-oxidative, anti-proliferative and phytoestrogenic) (Fig. 6.3) (Tvrdá et al. 2016).

Dietary QUE had a stimulatory effect on the hormone (FSH, LH and Prolactin) levels (at a concentration of 10, 100 and 1000 μg/kg body weight respectively) in the female rabbits (Tušimová et al. 2017). Similarly, Feeding with QUE improved

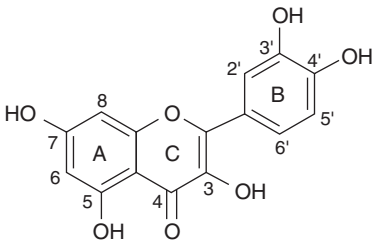

Fig. 6.3 Quercetin molecular structure

the folliculogenesis (increased the cell proliferation, weight of ovary, quality of oocyte and litter size) in the female mice with a reduction in cell apoptosis (Beazley and Nurminskaya 2016; Naseer et al. 2017). However, there is a suppression in the ovarian folliculogenesis and ovulation (also ovarian follicular atresia) in the (old) mice fed with QUE (Shu et al. 2011).

6.2.2.2 Molecular Targets of Quercetin

QUE is effective at concentrations of 0.3 and 30 μg/mL in the in vitro assay (Ader et al. 2000). However, the addition of QUE to the goat oocyte culture medium shows a dose-dependent activity: 4 μM of QUE improved the quality of oocytes (Silva et al. 2018) whereas 8 μM of QUE deteriorates (Orlovschi et al. 2014). Similarly, the addition of QUE at 25 μg/ml to the porcine oocyte culture medium showed an improvement in the oocyte maturation rates, blastocyst development with a cumulus cell expansion (Orlovschi et al. 2014). However, the addition of QUE (10 and 100 ng/ml conc.) to the porcine granulosa cell culture shown a reduction in the accumulation of proliferative (PCNA, cyclin B1) markers with the promotion of apoptotic markers (BAX) (Sirotkin et al. 2019).

Molecular targets of QUE is also having prime importance besides its dosage to use it as a safe and effective bioactive compound. QUE acts in multiple ways and it has a dose-dependent biphasic action on the ovarian functions (Hung 2007). In the swine granulosa cell cultures, QUE binds with estrogen receptors (ER β) and controls the release of 17β-estradiol (Krazeisen et al. 2001) in a dose-dependent manner. Indeed, at high concentrations, QUE had an inhibitory effect on 17β-estradiol, whereas at low concentrations stimulatory effect (Lu et al. 2012). Furthermore, studies conducted by Hung 2007 and Santini et al. 2009 revealed that QUE can inhibit VEGF development and thus prevent the angiogenic process. It can also stop steroidogenesis in granulosa cell cultures by suppressing the cytochrome P450 enzyme. Similarly, it can prevent aromatization in ovarian microsomes by altering cell signalling pathways and/or interfering with the NO/NOS system in granulosa cells (Santini et al. 2009). The above effects exerted by QUE indicates a negative effect of QUE on the ovarian physiology as it is inhibiting the process of angiogenesis, steroidogenesis as well as aromatization. Hence this distinctive property of QUE was being used extensively in ovarian cancer therapy (Hashemzaei et al. 2017). QUE has anticancer properties by regulating the cell cycle, modulating the

TGF1 factor (Scambia et al. 1994), inhibiting tumour development, angiogenesis, and inducing apoptosis in a dose-dependent manner (Parvaresh et al. 2016). QUE also controls the cell cycle by preventing the transition from the GO/GI to the G2/M process (Scambia et al. 1994). Furthermore, QUE also has an effect on aged oocytes, delaying postovulatory ageing in the oocytes by controlling SIRT and MPF (maturation promoting factor) activity (Wang et al. 2017a, b).

Aside from that, QUE also has an antioxidant impact (Wang et al. 2018). QUE is a bioflavonoid that is rich in antioxidants. QUE boosts step II antioxidant enzymes including superoxide dismutase (SOD), catalase, glutathione s-transferase (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx), and thioredoxin (Xu et al. 2019). Furthermore, it can also upregulate the Nrf2-ARE pathway similar as kaempferol (Saw et al. 2014).

Furthermore, QUE has its influence on hormones also. It regulates the gonadotropins (Shu et al. 2011), steroid and peptide hormones (Sirotkin et al. 2019). QUE mainly decreased the release of progesterone (P4) and Leptin with an increase in testosterone levels in the cultured granulosa cells (porcine) (Sirotkin et al. 2019). However, QUE harmed progesterone concentrations in the human granulosa cells, which could be due to the inhibition of the 3βhydroxysteroid enzyme (Lacey et al. 2005). QUE also had an effect on IGF-1 (insulin-like growth factor 1). Indeed, it has a dose-dependent effect on IGF-1 release in cattle granulosa cells: higher doses (100 ng/mL of QUE) inhibit, whereas lower doses (1 or 10 ng/mL) stimulate (Sirotkin et al. 2019).

6.2.2.3 Effect of QUE on Angiogenesis and Steroidogenesis

It is an undeniable fact that ovarian follicular development is mainly dependent on steroidogenesis and angiogenesis (Santini et al. 2009). Moreover, angiogenesis is mostly due to VEGF (vascular endothelial growth factor: bovines) and/or VEGFA (swine granulosa cells) (Robinson et al. 2009). QUE has nevertheless shown a significant impact on VEGF inhibitors, which in turn has also impacted proliferation, migration and differentiation in human and swine granulosa cells of endothelial cells (Santini et al. 2009). QUE has also been shown to inhibit steroidogenesis in humans (Rise et al. 2006) and porcine granulosa cells in a dose-dependent manner (Lacey et al. 2005). The suppressive effect of QUE on cytochrome P450 (which catalyzes the conversion of cholesterol to pregnenolone) could explain why QUE inhibits steroidogenesis (Rice et al. 2006). In human placental and ovarian microsomes, QUE also inhibits the aromatization process (androstenedione to estrone and testosterone to estradiol) (Kellis and Vickery 1984). Binding of QUE to estrogen receptors and/or modulation of cell signaling pathways (Whitehead and Lacey 2003) or interference with the NO/NOS system in swine could be the mechanism behind this inhibition.

6.2.2.4 Effect of QUE on Hormones

Dietary QUE had been a stimulatory effect on FSH, LH and Prolactin hormone levels in the plasma at a concentration of 10, 100 and 1000 μg/kg bodyweight respectively in the female rabbits (Tušimová et al. 2017). In the case of progesterone concentrations, QUE had a negative effect (Phytoestrogenic effect) in the human granulosa cells, which could be due to the inhibition of 3βhydroxysteroid enzyme (Lacey et al. 2005). However, on the estrogen (E2) concentration QUE had a dose-dependent biphasic activity (Santini et al. 2009).

6.2.2.5 Effect of QUE on Invitro Assays

QUE is considered as one of the potential modulators of ovarian functions. So also is used in the regulation of ovarian functions (ovarian folliculogenesis, oocyte maturation and ovulation) as well as treatment of reproductive disorders (Santini et al. 2009). However, QUE appears to be effective at 0.3 and 30 μg/mL for invitro assay as a beneficial ingredient (Ader et al. 2000).

QUE has been proven as an effective bioflavonoid for the IVM medium of bovine (Guemra et al. 2013). Inclusion of QUE (25 μg/ml) in the porcine oocyte culture medium resulted in the improvement of oocyte maturation rates, blastocyst development and cumulus cell expansion by reducing ROS levels at intracellular (Orlovschi et al. 2014).

Similarly, the addition of QUE at a concentration of 4 μM to goat oocyte culture medium (IVM medium) improved the quality of oocytes (Silva et al. 2018) by enhancing the mucification process, mitochondrial activity as well as by lowering the DNA fragmentation (Tarazona et al. 2006). However, the addition of 8 μM of QUE to the same medium lowers the cumulus expansion rates with a reduction in the oocyte maturation (Orlovschi et al. 2014). The decrease in synthesis and/or release of 17-estradiol due to QUE (at 8 μM) binding with oestrogen receptors (E2) is thought to be the cause of oocyte retraction (Kubo et al. 2015). Other potential causes include changes in oocyte function and a decrease in granulosa cell growth factor synthesis (Santini et al. 2009). Similarly, Jia et al. (2011) discovered that growing chicken follicular cell growth by adding QUE may be due to a reduction in apoptotic cells.

6.2.2.6 QUE as an Antioxidant

In menopausal rats, QUE protects the ovaries from cell viability decline caused by H_2O_2-induced oxidative stress and increases the expression of oxidative stress-related proteins (Wang et al. 2018). Furthermore, the inclusion of catechol and the –OH group in QUE contributes to its anti-oxidative properties by effectively scavenging free radicals.

QUE is considered as one of the effective radical scavenging as well as metal chelating bioactive flavonoid due to the presence of catechol (B ring) and a hydroxyl group (A ring) in its structure (Heijnen et al. 2002). The mechanism by which QUE can exert its antioxidant property is by increasing the antioxidant enzymes (GSH, SOD levels) (Kobori et al. 2015) and/or by activating the PI3K/Akt signalling pathway (Braga et al. 2019) in diabetic rats. Similarly, the addition of QUE at a concentration of 4 μM to the IVM medium protected the cell from damage, lessen the rate of apoptosis which resulted into a good quality of oocytes (Crocomo et al. 2013). However, 8 μM of QUE in the IVM medium led to decreases in GSH and mitochondrial levels in the cells (Silva et al. 2018). The reason behind this declinement attributed to being a high concentration of QUE, which can increase the intracellular calcium levels by disrupting the activity of Ca2 + -ATPase pump which in turn damages the mitochondria (Tvrdá et al. 2016).

6.2.3 Myricetin

Myricetin (MYR) is a polyphenolic bioflavonoid. MYR is a beneficial compound which is having antioxidant, antiangiogenic, anti-inflammatory as well as antineoplastic properties.

6.2.3.1 Structure

MYR is a 3,5,7,3′,4′,5′-hexahydroxyflavone in structure. The pyrogallol B-ring distinguishes myricetin, and its more hydroxylated form is thought to be responsible for its improved biological properties as compared to other flavonols (Fig. 6.4). Myricetin-3-O-(3′′-acetyl)—L-arabinopyranoside, myricetin-3-O-(4′′-acetyl)—L-arabinopyranoside, and myricetin-3-O—L-rhamnopyranoside are examples of free and glycosidically linked types (Taheri et al. 2020).

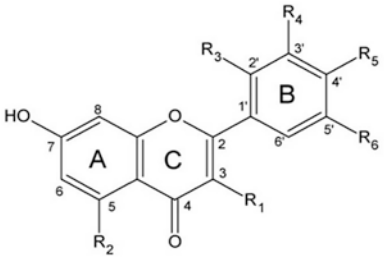

Fig. 6.4 Myricetin molecular structure

6.2.3.2 Molecular Targets

MYR has estrogenic properties as well. In Wistar albino rats, oral administration of MYR at 100 mg/kg/day resulted in estrogenic activity. The uterus's height and weight were elevated, triggering this event (Karabulut and Barlas 2018).

The difference in the synthesis of reactive oxygen species (ROS) and antioxidant enzymes can wreak havoc with ovarian functions (oocyte maturation, ovulation, fertilization, implantation, and embryo development), which is undeniable (Wang et al. 2017a, b). MYR may also defend cells by restoring the activity of antioxidant response enzymes (Catalase, glutathione peroxidase and Superoxide dismutase) and increasing its protein expression (Wang et al. 2010). Besides that, by regulating the PI3K/Akt and MAPK signalling pathways, MYR can protect cells from oxidative stress-induced apoptosis (Kang et al. 2010).

MYR also functions as a chemoprotective compound against cancer cells by modifying the several distinct pathways (apoptosis, angiogenesis, cell proliferation, signalling pathways, and tumour metastasis). MYR inhibits cancer cell neoplasia by interacting with oncoproteins such as Protein Kinase B (PKB)/(Akt), Fyn, MEK1, and JAK1– STAT3 (Devi et al. 2015). MYR also prevents cancer cell angiogenesis through the p21 (Cyclin kinase inhibitor-1)/HIF-1 (Hypoxia-induced factor)/VEGF pathway (Huang et al. 2015). Similarly, MYR inhibited angiogenesis in A2780/CP70 and OVCAR-3 cancer cells by inhibiting Vascular endothelial growth factor and/or reducing the levels of p-Akt, pp70S6K, and HIF-1 factors (Huang et al. 2015).

6.2.4 Curcumin

Curcumin is a phenolic bioactive compound concealed in the rhizomes of *Curcuma longa*. Curcumin's pro-apoptotic, anti-proliferative, anti-oxidant, and anti-angiogenic abilities have made it beneficial in the treatment of a variety of diseases, including cancer (Tiwari-Pandey and Ram 2009).

6.2.4.1 Structure

Chemically curcumin is a 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione with a formula of $C1_2H_{20}O_6$ (Fig. 6.5) (Aggarwal et al. 2005).

6.2.4.2 Molecular Targets of Curcumin

Curcumin acts in the ovarian cells by up regulation of transcription factors (Nuclear factor erythroid 2-related factor 2-Nrf2) and their regulated genes (Heme oxygenase-1) (Yan et al. 2018). Additionally, curcumin also acts by decreasing the apoptotic related proteins (caspase-3 and -9) and genes (Bad and Bax) which is majorly

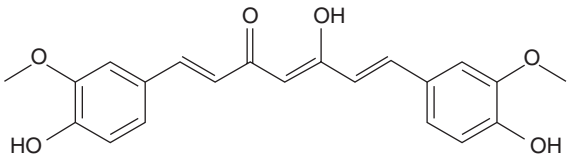

Fig. 6.5 Cucumin molecular structure

due to the up regulation of PI3K and AKT pathways (Atif et al. 2015). Furthermore, being a phytoestorgenic compound, curcumin can exerts its effects on hypothalamo–hypophysial–ovarian axis by interacting with endocrine system (Bachmeier et al. 2010) also. Similar way curcumin can also downregulate the androgen receptors with a upregulation of 3-β-hydroxysteroid dehydrogenase enzyme levels in the curcumin induced mouce ovaries was reported by Tiwari-Pandey and Ram 2009.

6.2.4.3 Effects of Curcumin on Ovarian Functions

Curcumin has the ability to shield the ovaries from ageing, ovarian insufficiency, and/or oxidative stress (Alekseyeva et al. 2011; Qin et al. 2015). Curcumin has a stimulatory effect on ovarian functions, facilitating reproduction and reducing apoptosis in a variety of species (Swine: Nurcahyo and Soejono 2007; Murine: Aktas et al. 2012). Curcumin has also been shown to improve the processes of ovarian folliculogenesis, steroidogenesis, and oogeneis in rodents (Alekseyeva et al. 2011). Curcumin, on the other hand, decreased fecundity and suppressed growth and progesterone release in rat ovarian luteal cells, resulting in a further delay in puberty (Murphy et al. 2012). Curcumin inhibits replication, promotes apoptosis, and suppresses progesterone and esradiol release in porcine ovarian granulosa cells (Nurcahyo and Soejono 2007), which has been well established. However, curcumin reduced the fecundity suppresses the proliferation and progesterone release with a further prolongation of puberty in the rat ovarian luteal cells (Murphy et al. 2012). Similarly, Curcumin inhibited the proliferation, enhances the apoptosis and suppresses the progesterone as well as estradiol releases in the porcine ovarian granulosa cells (Nurcahyo and Soejono 2007) also well documented.

Addition of curcumin @20 μM to the bovine COCs culture has improved the expression levels of GDF-9 and KitL which subsequently improved the folliculogenesis. Likewise, curcumin @36 and 48 mg/kgbw/day increased the folliculogenesis and oocyte quality in the mice having ectopic endometrirtis through the inhibitory pathways of TNF-α secretion. Curcumin also activated ovarian activity and facilitated folliculogenesis in murine ovarian cells (Aktas et al. 2012). Yan et al. 2018 reported an improvement in total follicle number (primordial, major, and secondary) after intraperitoneal injection of curcumin to the mouse (100 mg/kg/day for 42 days) through the Nrf2/HO-1 and PI3K/Akt signalling pathways. Likewise, 100 μg/g doses of cucumin has rescued the ovaries from dysfunction by blocking

the potent inflammatory factor NF-κB in the mice. However, Kizilay et al. 2017 reported the anti implantation and anti proliferation activity of curcumin in the endometritis model rats. Likewise, Vashist et al. 2018 reported the loss of granulosa cell viability by the addition of curcumin at 50 μM in buffalo granulosa cells. Similarly, Moreira-Pinto et al. 2019 reported a cytotoxic effect of curcumin @50 μM doses (24 h and 48 h) and 10 μM doses (72 h) in the human granulosa cells. Likewise, addition of curcumin downregulated the proliferation of ovarian granulosa cells reported by Hanif et al. 1997. However, Shi et al. 2006 reported this activity as beneficial in human ovarian cancer cells as incorporation of curcumin in the regular diet diminished the proliferation of human ovarian cancer cell. Similalry, in the human granulosa cells curcumin at lower doses (0.001 μM) reduces the ROS/RNS generation where as at higher doses (5 μM) ROS/RNS production was increased (Moreira-Pinto et al. 2019). Yet another instance reported by Kádasi et al. 2017 as curcumin inhibited the porcine granulosa cell proliferation which has been evidenced by caspase activation.

6.2.5 Resveratrol

Resveratrol is a natural polyphenol with antioxidant, anti-inflammatory, and anti-aging properties (Donnez et al. 2009).

6.2.5.1 Structure

Resveratrol is a polyphenolic dietary stillben (3,5,40-trihydroxystilbene). In fact, there are two isoforms of Resveratrol: Trans and Cis Resveratrol, with the Trans form becoming more common in plants (Fig. 6.6) (Camont et al. 2009). Resveratrol is made from phenylalanine by activating the stilbene synthase enzyme (Donnez et al. 2009).

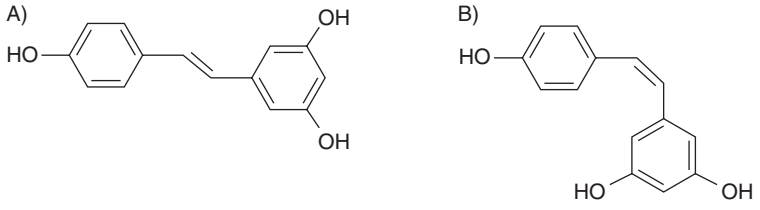

Fig. 6.6 Resveratrol molecular structure: (**a**) Trans (**b**) Cis

6.2.5.2 Molecular Targets of Resveratrol

Inhibition of thePI3K/Akt Signaling Pathway

This pathway is a serine/threonine dependent and responsible for the regulation of cell proliferation as well as survival. Resveratrol inhibits estrogen sensitive cell survival and proliferation by interfering with the estrogen receptor associated PI-3 K pathway (Pozo et al. 2004).

Inhibition of Nuclear Factor- B

Nuclear factor-B (NF-κB) is a transcription factor that plays an important role in cell proliferation, differentiation, and carcinogenesis. Resveratrol's above-mentioned effects are primarily accomplished by inhibiting NF-κB, which has been activated by tumour necrosis factor α (Manna et al. 2000). Furthermore, by implicitly triggering SIRT1, resveratrol has a negative feedback influence on the FSH/NF-B mechanism (Pavlova et al. 2013).

Activation of Sirtuins

Sirtuins are a family of nicotinamide adenine dinucleotide–dependent deacetylases that play a role in genomic stabilisation, DNA repair, transcriptional silencing, and lipid metabolism, among other things (Qiang et al. 2011). Via the activation of SIRT1, resveratrol prevents oocytes from ageing. SIRT1 is expressed in the ovary's oocytes and granulosa cell nuclei at different stages of follicular growth (Chen et al. 2010). Sirtuins are also important regulators of granulosa cell apopotosis, terminal differentiation, and steroidogenesis (Morita et al. 2012).

Both caspase-3/7 activation and DNA fragmentation detection revealed that resveratrol induced a concentration-dependent increase in apoptosis. Resveratrol also caused morphological changes in theca interstitial cells that were time and concentration dependent, consistent with apoptosis, such as nuclear shrinkage with condensed chromatin and cytoskeleton degradation/fragmentation (Wong et al. 2010). As a result, resveratrol's proapoptotic effects on the theca-interstitial compartment have been observed at various stages of the apoptotic cascade.

6.2.5.3 Phytoestrogenic Actions

Resveratrol has phytoestrogenic properties due to its chemical structure's resemblance to estrogens such as diethylstilbestrol (DES) (Gehm et al. 1997). Since it has two six-carbon rings with hydroxyl side chains, resveratrol may be mistaken for estradiol. As a result, Resveratrol acts as an agonist for the two estrogen receptor subtypes (ER α and ER β) (Baker and Lathe 2018).

In several studies, it has been shown that resveratrol modulates both female and male reproduction. In females, resveratrol is considered a phytoestrogen with a chemical structure similar to that of some estrogens. Interestingly, resveratrol is potentially usable alone or in combination with other hormones for its moderate estrogenic effect. Moreover, resveratrol exerts a steroidogenesis modulation in the ovary via sirtuins, especially SIRT1. Finally, resveratrol is a quality enhancer of aged oocytes and a gametes cryo-protectant, with mainly antioxidant and anti-apoptotic effects. In males, resveratrol modulates the reproductive function by: (1) enhancing the production of testosterone, (2) triggering penile erection, and (3) improving spermatogenesis including sperm differentiation and number in the testes and ejaculate, respectively. The mechanisms of action seem to be exerted by activating the AMPK pathway. Finally, resveratrol is a suitable antioxidant to supplement to semen extenders thanks to its beneficial effect in preserving sperm quality. However, although considerable research supports the positive impact of resveratrol on human and animal reproduction, further studies are necessary to consolidate the knowledge on the properties of resveratrol and its role in the reproductive functions.

6.2.5.4 Effects of Resveratrol

Resveratrol improves the quality and maturation of mature oocytes as they get older (Sugiyama et al. 2015) via SIRT1-related cellular pathways. In fact, by activating either folliculogenesis-related molecule and/or gonadotropin receptors, Resveratrol and SIRT1 have the ability to modulate ovarian functions. SIRT1 can bind to genes involved in energy homeostasis, cell survival, gene silencing, and genomic stability (NF-kB, FOXO4, HIF 2 alpha, FOXO1,. Likewise, resveratrol also increased SIRT1 levels in porcine granulosa cells, resulting in a faster apoptotic rate and follicular atresia (Zhao et al. 2014). Incorporating Resveratrol into porcine granulosa cell culture media had a similar effect, inhibiting cell proliferation while increasing the apoptotic rate (Sirotkin 2016). By enhancing the levels of SIRT1 in the granulosa cells, cumulus cells, oocytes, and blastocysts, resveratrol increased oocyte maturation and embryonic growth in bovines (Wang et al. 2014). Inclisuion of resveratrol (20 M) to the IVM medium improves GSH levels in cattle oocytes (Sovernigo et al. 2017) also.

Resveratrol increases the embryo developmental competence to the subsequent blatocyst stages (Lee et al. 2010). The embryos obtained by IVF with a Resveratrol treatment (0.5 μM) in the porcine exerted a positive effect by increasing the blatocyst cell number and decreasing the apoptotic related genes (BCL2, caspase-3) expression (Lee et al. 2010). In a similar case porcine embryos supplemented with resveratrol (2.0 μM) during in vitro maturation showed increased developmental capacity by rising intracellular GSH levels and decreasing ROS levels (Kwak et al. 2012). Similarly, addition of resveratrol (0.5 μM) to the IVC medium by achieving the higher hatching rates (Salzano et al. 2014).

6.3 Conclusion

Administration of plant extracts and/or active biomolecules as a source of drugs in the treatment of human and/or animal diseases has been a traditional practice since antiquity. Plants and/or plant-based medicines are increasingly being used in the treatment of various reproductive disorders (fertility, ovarian dysfunctions, etc.) due to their abundant supply, low cost, and lack of side effects. Furthermore, synthetic chemical medications (medicines, hormones, growth promoters, and so on) can be substituted with the natural plant sources for the desired development or reproduction in the mammals. In the field of plant-based therapy for animals, phytobioactive compounds (Kaempferol, Quercetin, Myricetin, Curcumin, and Resveratrol) are the main chemicals responsible for modulating ovarian functions. They carry out the activities of ovarian cells both in vivo and in vitro by targeting hormones, enzymes, reactive oxygen species, and several other molecules. It is, however, dose-dependent. Furthermore, the compounds mentioned above can be dosed in a number of ways, including crude extract, active compound, essential oil, and so on. As a whole, precise molecular targets of bioactive compounds are needed to enhance the reproductive ability of mammals using plant-based therapy.

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Chapter 7
Buffalo Embryo Production

Qaisar Shahzad, Muhammad Waqas, and Yangqing Lu

Abstract The domestic buffalo, *Bubalus bubalis*, has gained an increasing demand for in vitro embryo production technologies in the last few years for faster propagation of superior germplasm. This demand is explained by the low efficiency of multiple-ovulation, adverse effects of heat stress, less survival rate post-vitrification, not well-defined *in vitro* culture media, and embryo transfer programs. The early procedures for the buffalo in vitro embryo production were borrowed from the techniques being efficiently used in cattle. The progressive availability of more specific information about the *in vitro* culture requirements of the buffalo oocyte and embryo has improved the efficiency over the years. Although the figures of *in vitro* embryo production have attained a rise, which is indicated by competitive embryo yields; pregnancy rate and development to term are still poor. This all emphasizes the demand for the optimization of embryo cryopreservation methods. This chapter reviews in vitro embryo production in buffalo species.

Keywords Buffalo · Ovum pick up · Serum free culture media · Proteomics

Abbreviations

6-DMAP	6-dimethylaminopurine
ACAT2	Acetyl-CoA Acetyltransferase 2
ACSL4	Acyl-CoA Synthase 4
BO	Brackett Oliphant
BSA	Bovine Serum Albumen

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COCs	Cumulus Oocytes Complexes
CRISPR Cas-9	Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9
E2	Estradiol
ER	Endoplasmic Reticulum
FAOSTAT	The Food and Agriculture Organization Corporate Statistical Database
FSH	Follicle-Stimulating Hormone
ICSI	Intracytoplasmic Sperm Injection
iTRAQ	isobaric Tag for Relative and Absolute Quantitation
IVC	*In Vitro* Culture
IVEP	*In Vitro* Embryo Production
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Maturation
KEGG	Kyoto Encyclopedia of Genes and Genomes
MEM	Minimal Essential Medium
MOET	Multiple Ovulation and Embryo Transfer
MS	Mass Spectrometry
SCNT	Somatic Cell Nuclear Transfer
SOF	Synthetic Oviductal Fluid
TALP	Tyrode Modified Medium
TCM	Tissue Culture Medium
TG	Triglycerides

7.1 Introduction

Buffalo is the 2nd largest milk-producing animal with a contribution of 13.2% in the total milk produced all around the world, while cattle being first with an 83% share in overall milk production (FAOSTAT 2015). Major buffalo milk producers (97%) exist in Asia including India and Pakistan at the top. During 2014 in Italy, the buffalo population attained a tremendous rise from 185,000 in 2004 to 369,352. Almost all the milk (200,000 t/year) in Italy is processed into mozzarella cheese, a typical fresh cheese that has a high market value (FAOSTAT 2015). This makes the buffalo milk price about thrice higher than that of milk of dairy cattle (Correddu et al. 2017). Generally, buffalo are considered as low milk producers but it has the incredible potential of producing more than 5000 liters/lactation (Bilal et al. 2006).

However, buffaloes exhibit low reproductive performance and demonstrate inherent reproductive problems. These include seasonality of breeding, silent estrus signs, delayed onset of reproductive maturity, low number of primordial follicles, and late post-partum conception have been attributed to poor reproductive performance of this species (Nandi et al. 2002). But, to sustain the livestock production

system reproductive efficiency must be greater. As the increased world population has placed considerable challenges for food security, therefore, an increase in productivity is imperative to curb the food scarcity problem in the future. The advent of modern reproductive biotechnologies has opened avenues to manipulate and enhance reproductive performance in various animals (Choudhary et al. 2016). The various developments in the field of buffalo embryo technology are discussed below.

7.2 Influence of Seasonality on Buffalo Embryo Reproduction

Certain environmental issues contribute to influencing the reproductive activity of buffalo while nutrition and photoperiod impart their influence to different degrees which are dependent upon the geographical areas (Gasparrini 2019). Buffalo exhibits seasonal anestrus because of the heat stress in certain countries which are present in the north of the Equator. These include India, south China, Pakistan, and Egypt. A country like Italy is the place where photoperiod is the main contributing factor. It is important to mention that heat stress plays its role by further contributing to the seasonal anestrus in countries like Pakistan, south China, India, and Egypt (Di Francesco et al. 2012).

In abattoir-derived ovaries, a decrease in the follicle and oocyte population was observed during summer by scientists from Egypt. They primarily attributed the influence of season to heat stress (Abdoon et al. 2014). However, it is also noted that the summer season vigorously impairs oocyte competence by decreasing the maturation, cleavage, and blastocyst rates. It also imparts its influence upon the gene expression level.

HSP70 is well known for its role as a proapoptotic factor. When there is an unfavorable period for the oocytes, this gene gets up-regulated in both IVM oocytes and cumulus oocytes complexes (COCs) (Abdoon et al. 2014). This helps to conclude that heat stress plays the main role in seasonality in buffalo reproduction. Season has also been proven to have a significant influence upon the success of achieving successful oocyte competence used for somatic cell nuclear transfer (Zheng et al. 2020). When COCs were exposed to a high temperature, the expression level of HSP70 got an increase in its trend (Payton et al. 2011). Buffalo oocytes matured *in vitro* in the non-reproductive season also showed destruction of cortical granules (Payton et al. 2004) and premature cellular senescence caused by accelerated cytoplasmic maturation in bovine oocytes exposed at 41 °C during IVM (Edwards et al. 2005).

A study was conducted to compare the influence of seasonality on buffalo oocyte maturation and blastocyst rate (Shahzad et al. 2020a). A decline in oocyte maturation and embryo development rate was observed when day length was increased during early summer season (April–June) as described in Fig. 7.1. In turn, the

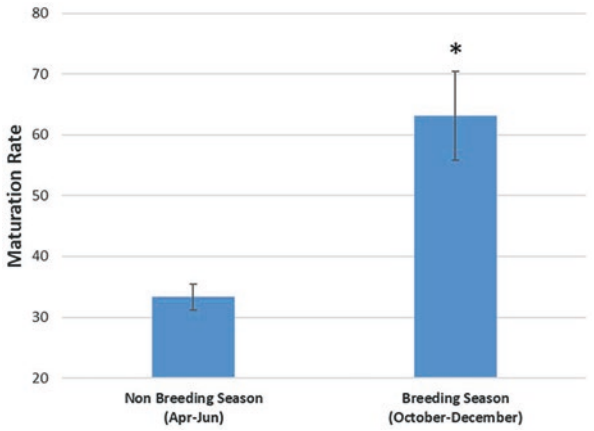

Fig. 7.1 Maturation rate in different seasons in relation to day light hours. Asterisk (*) indicates significant difference between the groups ($P < 0.05$). (This figure is reproduced from Shahzad et al. (2020a, b, c) with permission)

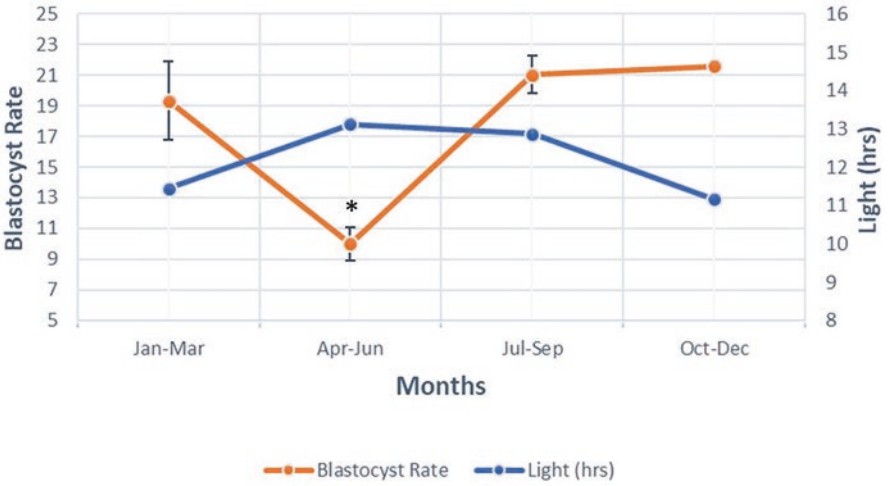

Fig. 7.2 Blastocyst rate in different seasons in relation to day light hours. Asterisk (*) indicates significant difference between the groups ($P < 0.05$). (This figure is reproduced from Shahzad et al. (2020a, b, c) with permission)

oocyte developmental potential and embryo quality was substantially improved during the rest of the year. A peak in oocyte maturation and blastocyst rate was observed during the autumn season (October–November) (Figs. 7.1 and 7.2).

So, it can be withdrawn from the above discussion that photoperiod and heat stress during non-breeding season is associated with a decline in the efficiency of embryo production. This makes it compulsory to perform ovum pick up (OPU) during the months of peak production (October–November). It is suggested that to overcome the influence of non-breeding season, melatonin and coagulansin-A can be added in the oocyte maturation media.

7.3 Multiple Ovulation and Embryo Transfer

To take advantage of the genetic potential of the females exhibiting elite phenotype, multiple ovulation and embryo transfer (MOET) technology has been developed. It requires superovulation, sperms insemination, and flushing of donors; and then for further use or to the transfer of embryos to the recipients, cryopreservation is done. In the USA, the first successful live buffalo calve has taken birth through using this technology (Drost et al. 1983) which has created substantial interest in buffalo rearing countries. But sill this technology involves certain limitations as mean embryo recovery after multiple ovulation (MO) followed by artificial insemination (AI) is less than 2 embryos which are estimated to be 5 embryos per animal in cattle. Such low responsiveness is considered to the rather less population of follicles which is 1/5th of the cow and to the poor quality of buffalo oocytes (Zicarelli et al. 1997).

7.4 In Vitro Embryo Production

An inconsistent efficiency of MOET in the buffalo has led to a worldwide raising concern at a large scale for the *in vitro* production of buffalo embryos. The main objective behind this lies in the faster propagation of superior germplasm. This will also help to enhance genetic progress through maternal contribution. Using slaughterhouse-derived oocytes for *in vitro* embryo production (IVEP) procedures has gained remarkable advancement in recent years. IVEP contains the potential to help to extend the germplasm of both male and female animals which are especially genetically superior. There are four major steps involved in the IVEP system. These include (1) collection of the oocytes from ovaries, (2) in vitro maturation (IVM) of these oocytes, (3) *in vitro* fertilization (IVF) of these *in vitro* matured oocytes, and then at last (4) *in vitro* culture (IVC) of fertilized oocytes till the blastocysts stage.

The application of IVEP describes the role of biotechnology to increase embryo production with greater reproductive potential and the dissemination of selected germplasm in buffalo. However, other peculiarities including the low number of ovarian follicles and viable oocytes recovered and the effects of seasonality on oocyte quality which are inherent to this species have limited the use of this biotechnology in buffalo. Let's take into detail the description of IVEP practiced in buffalo.

7.4.1 Collection of Oocytes

There are mainly two sources of oocytes; oocytes retrieved from the ovaries from a slaughterhouse or by using OPU from the live animals. OPU has an advantage over abattoir ovaries as the genetic potential of elite females may be utilized by using this technique. OPU is superior to slaughterhouse ovaries because the genetic potential of females can be harnessed through the use of this technology. Currently, with the evolution of ART, it is possible to establish efficient programs for the use of embryo production and transfer in buffalo. The application of OPU/IVEP is showing promising results, and has become an alternative to superovulation for in vivo embryo production. Furthermore, several factors shown to be critical for efficacious OPU/IVEP, include circulating levels of AMH, AFP, size of the follicles available for the OPU procedure, reproductive seasonality, semen (sire) used for IVEP, donor category and farm. The use of OPU/IVEP allows the rapid multiplication of high merit genetic material through both the female and male lineages (Baruselli et al. 2020). In srecent years, the method of using slaughterhouses as oocytes has made enormous progress. This technology is very important for the mass production of embryos for research purposes (Madan et al. 1994; Totey et al. 1996; Chauhan et al. 1998), genetic manipulation and the improvement of animal populations (Sagirkaya et al. 2004), embryo transfer as well as commercial purpose (Nandi et al. 2002).

Techniques need to be developed that help recovers large numbers of oocytes in each ovary. The total number of oocytes obtained from each ovary varies depending on the method of retrieval. Oocytes were collected via slicing, follicle puncture, and follicle aspiration to collect oocytes. Slicing has been reported to be the best method of egg collection in general, but is time-consuming and impractical and can cause the COC to lose vitality (Das et al. 1996). Therefore, the aspiration method is usually used for feasible COC collection. The OPU allows for the continuous retrieval of competent oocytes. Recent studies have shown that performing the OPU every 14 days can better produce high-quality embryos (Konrad et al. 2017).

7.4.2 In Vitro Maturation

The ability of immature buffalo oocytes aspirated from slaughtered buffalo ovaries to mature in vitro is largely influenced by the maturation medium and supplements. Buffalo‘s IVM uses a variety of Ham, MEM, and TCM-199, F-10 media (Samad et al. 1999; Hussain et al. 2005) but TCM-199 is considered the most commonly used media for bovine oocyte (Stagmiller 1988). Its varying effects of the composition of media on IVM, IVF, or its further development have also been demonstrated (Bavister et al. 1992).

The effect of hormones on the growth and development of buffalo follicles was investigated and follicle-stimulating hormone (FSH) and estradiol (E2) were found to be important components of IVM (Totey et al. 1992). Furthermore, luteinizing

hormone (LH) is crucial in IVM, fertilization, and development. In another study, FSH, LH, and E2 were reported to cause a synergistic enhancement of nuclear maturation and expansion of buffalo oocyte heap cells (Totey et al. 1993; Jamil et al. 2007). Some previous studies have shown that adding a serum to the in vitro culture medium, IGF-1 and EGF can promote the maturation of oocytes (Lorenzo et al. 1994; Kumar and Purohit 2004; Lonergan et al. 1996) and also influence subsequent development in vitro (Herrler et al. 1992). Somatic cells, such as cumulus or granulosa cells, have been shown to support the growth of buffalo oocytes and embryos. (Nandi et al. 2002).

The duration of the IVM plays a crucial role because inappropriate maturation time can cause chromatin abnormalities (Dominko and First 1997). The best time for in vitro fertilization (IVF) is the time when meiosis is completed. Meiosis occurs at different times in different species. The time for cattle is 18–24 h (Sirard et al. 1989; Neglia et al. 2001) and the time for pigs is 36–48 h (Prather and Day 1998). Although large differences in oocyte maturation time have been reported in buffaloes, the highest percentage of MII oocytes was observed between 16 and 24 h (Neglia et al. 2001; Yadav et al. 1997; Gasparrini et al. 2008).

7.4.3 In Vitro Fertilization

In water buffaloes, fertilization is generally considered to be the most critical step in the IVEP procedure (Neglia et al. 2003; Gasparrini et al. 2004a). Media commonly used for in vitro fertilization of buffalo are Tyrode Modified Medium (TALP) and Brackett Oliphant (BO), supplemented by factors that induce sperm motility. In direct comparison experiments, the TALP medium supplemented with heparin, hypotaurine, and penicillin was used to obtain higher cleavage and blastocyst rates (Gasparrini et al. 2004a).

The time of co-incubation of the gametes is very important. The optimal egg-sperm co-incubation time to maximize buffalo blastocyst production is 16 h (Gasparrini et al. 2008). Shortening the gamete co-incubation time to 8 h can significantly reduce oocyte lysis (Ward et al. 2002; Kochhar et al. 2003). Conversely, extending gamete co-incubation to 20 h is detrimental because blastocyst production is reduced and multiple spermatozoa are observed (Sumantri et al. 1997).

7.4.4 In Vitro Culture

A large amount of evidence provided in the last decade shows that the development potential of embryos in vitro depends mainly on the quality of the oocyte from which the embryo originated (Sirard et al. 2003). It is also influenced by the cultural environment in which the embryo is located (Lonergan et al. 1999; Van Soom et al. 2002; Yuan et al. 2003). To improve embryo dynamics and blastocyst rate in IVP,

serum at a concentration of 1–20% (v/v) is added to the embryonic medium (Holm et al. 2002). The serum contains beneficial substances that are beneficial for embryonic development, including growth factors, heavy metal chelating agents, and components that cause cumulus cell expansion, even if FSH is not administered externally (Abe and Hoshi 2003). Studies have also proved that progesterone (P_4) supplementation during in vitro embryo culture helps to significantly improve the blastocyst rate (Pandey et al. 2020).

Although fetal bovine serum (FBS) is added as energy source and growth factor, there is a risk of pollution and can cause abnormal fetal development, such as poor implicit memory (Fernández-Gonzalez et al. 2004) and large offspring syndrome (Lazzari et al. 2002). Serum supplementation can also cause lipid accumulation in cells, making embryos easier to cryopreserve (Abe and Hoshi 2003; Diez et al. 2001). Triglycerides (TG) are produced by the endoplasmic reticulum (ER) and stored in cytoplasmic lipid droplets and are the most abundant lipids in oocytes and embryos (Ferguson and Leese 1999; Stone et al. 2009). It has been shown that decreasing FBS concentration during in vitro maturation (IVM) can reduce the accumulation of embryonic lipids without affecting the incidence of blastocysts (Del Collado et al. 2016). Transcriptomic analysis of the embryos in the absence of serum showed that they were more similar to derived embryos in vivo (Heras et al. 2016).

Somatic cells contained in the preimplantation embryo culture medium (co-culture) have effects on the nutrition of the embryo, such as faster cleavage (Bongso et al. 1989), increased blastocyst cell count (Smith et al. 1992), increased blastocyst rate (Rexroad Jr 1989), a decrease in apoptosis (Xu et al. 2000), enhanced pregnancy rates (Wiemer et al. 1989) and live births (Marcus and Brinsden 1996). However, communism is a double-edged sword, as long as it has many harmful effects in the postpartum period (Orsi and Reischl 2007). Somatic cells are more metabolically active and show health problems (Goovaerts et al. 2011). In co-culture experiments, somatic cells change their natural appearance by forming a monolayer at the bottom of the petri dish and may lose their aromatase activity due to luteinization (Goovaerts et al. 2011; Li et al. 2000).

Currently, with the development of ART, effective procedures can be established to utilize the production and transfer of buffalo embryos. The application of IVEP has shown promising effects and has become an alternative to superovulation. Additionally, several factors that have been shown to be critical for the efficacy of IVEP include AMH, follicle size available, reproductive seasonality, semen used for IVEP (father), donor category, and breeding. The use of IVEP enables the rapid propagation of high-value genetic material through female and male lineages. Therefore, embryo transfer can be implemented commercially, combined with the genetic improvement and increased reproductive efficiency, to increase the meat and milk production of farmed buffalo.

7.5 Establishment of Serum-Free Culture Media

Powerful serum-free culture system, consisting of synthetic oviductal fluid (SOF), bovine serum albumin (BSA), and insulin transferrin-selenium (ITS). Compared to the serum-supplemented medium, the blastocyst rate (~ 40%)) is considerable. The hatching rate of bovine embryos produced under these conditions without serum is much lower than that of embryos produced in serum-containing media (George et al. 2008; Wydooghe et al. 2014). However, in many other respects, the quality of embryos produced in the absence of serum is better than the quality of embryos produced in the presence of serum. Embryos produced under serum-free conditions show increased freezeability. After transfer, the birth weight and abnormality rate of the calf produced are consistent with the birth weight and abnormality rate of the derived embryos in the body (George et al. 2008; Young et al. 1998). Embryos produced under serum-free conditions show gene expression patterns more similar to those in vivo produced embryos (Heras et al. 2016).

Furthermore, when traditional parameters are used to assess embryo quality, bovine embryos produced under serum-free conditions score higher (George et al. 2008; Wydooghe et al. 2014). Similarly, buffalo embryos were grown in a serum-free culture system for cattle. It was revealed that the egg maturation medium should contain serum and the medium should exclude it so that the buffalo embryos can develop better (Shahzad et al. 2020b). A blastocyst rate of 43% has been obtained by using serum-free culture media for in vitro production of buffalo embryos at Guangxi University (Unpublished data). All the results of above-mentioned studies are strong and the abnormal effects of adding serum to embryos medium are well-documented. Therefore, the IVF medium should be switched to serum-free supplements, including for research as well as commercial purposes.

7.6 Cryopreservation of Embryos

Cryopreservation is a technique that can be used to support the buffalo embryo marketing plan because it can gain flexibility in embryo transfer plans by allowing embryo transfer in a more favorable season of the year and simplifying the transport and sale of genetic material (Mandawala et al. 2016). Several cryopreservation methods traditionally used for other familiar species, namely slow freezing and vitrification, have been tested on oocytes and embryos of buffalo species (Parnpai et al. 2016). However, the specificity mainly linked to the content and composition of cytoplasmic lipids of gametes and embryos of this species (Boni et al. 1992) hindered the progress of the results.

However, some factors can influence the response of embryos to the cryopreservation process. It is emphasized that the developmental stage of the embryo, the composition of the vitrification solution and the equilibrium time used, as well as the origin of the embryo (in vivo or in vitro), and the morphological quality of the

structure are the reasons for the difference in the development of the embryo results (Parnpai et al. 2016).

Traditionally, a combination of cryoprotectants with and without the ability to penetrate cytoplasmic membrane cells has been used in vitrification protocols. Yang et al. (Yang et al. 2012) tested different combinations and times and found that the best results were obtained when the embryos were cultured in vitro for 6–7 days and vitrified in a solution containing the following ingredients: 20% ethylene glycol +20% DMSO +0.5 M sucrose.

The addition of chemicals affecting energy metabolism to the culture medium has been evaluated as a viable option to improve the vitrification efficiency of mammalian embryos (Held-Hoelker et al. 2017). The addition of L-carnitine during the in vitro culture of buffalo embryos has shown an increase in survival after devitrification (Verma et al. 2018). The mechanism linked to this positive reaction, in addition to its antioxidant capacity, is also linked to its role in increasing the oxidation of intracellular fatty acids (Abdelrazik et al. 2009), thus reducing the intracellular lipid content and protecting embryonic cells from oxidative stress.

A study by Shahzad et al. (2020c), concluded that KEGG analysis of differentially expressed proteins increased the expression of fatty acid degrading proteins (ACAT2 and ACSL4) in embryos grown under hypoxic conditions. These results are supported by the fluorescence intensity of low lipids in embryos under physiological oxygen. The intensity of the fluorescence is an indicator of the density of the lipid droplets (Verma et al. 2018). The higher lipid levels are attributable to the poor survival of embryos at low temperatures (Gasparrini 2002).

Proteomics and fluorescence intensity analysis indicate that the improved low-temperature survivability of hypoxic embryos may be due to lower lipid levels. Acetyl-CoA acetyltransferase 2 (ACAT2) is an enzyme that catalyzes a reaction that converts two acetyl Co-A to acetoacetyl Co-A (Merilainen et al. 2009). The long-chain member of the acyl-CoA synthase 4 (ACSL4) family is an enzyme that uses free long-chain fatty acids and converts them to fatty acyl-CoA esters, and thus plays a key role in the biosynthesis of lipids and the degradation of fatty acids. This enzyme preferentially uses arachidonic acid as a substrate (Kuch et al. 2014). Since arachidonic acid is a polyunsaturated fatty acid, it has been suggested that increased expression of the ACSL4 protein may increase the degree of unsaturation in the phospholipid bilayer of the membrane. However, it is recommended to use lipidomic methods to confirm this phenomenon.

7.7 Pregnancy Rate Following Transfer of In Vitro Fertilization Embryos in Buffalo

Recently, a Brazilian team achieved a pregnancy rate of around 22% after transferring fresh embryos produced in vitro (Marin et al. 2019). Similarly, China has also reported the transfer of fresh and frozen-thawed embryos produced in vitro, with

pregnancy rates of 44.4% and 33.3% respectively (Yangqing Lu and Kehuan Lu 2015). These studies demonstrated the feasibility of using OPU-IVEP-ET to expand the buffalo core. There is a strong relationship observed among the synchronization protocol, diameter of the CL and the season of embryo transfer. These parameters are strongly associated with the success of the embryo transfer and pregnancy rate in buffalo (Saliba et al. 2020). A number of approaches can be adopted to improve the success of embryo transfer. These may include the treatment strategies which can be helpful to improve the luteal function and ways to strengthen the quality and competence of oocyte. Semen quality and photoperiod also have a strong correlation with successfulness of embryo transfer (Gasparrini 2019). Interestingly, Buffalo IVF has recently been marketed in Brazil, India, and Italy.

7.8 Parthenogenetic Activation of Buffalo Oocytes

Parthenogenesis is a form of asexual reproduction that involves oocytes activated in the absence of sperm to produce non-viable blastocysts. Parthenogenetic embryos carry only maternal chromosomes and can be produced by inducing the oocytes to resume meiosis without fertilization. Methods such as electrical stimulation using chemical reagents (such as calcium ionophore, ethanol, strontium chloride, phorbol ester, thimerosal, and phospholipase zeta) have been used successfully to activate bovine oocytes (Ross et al. 2008). The parthenogenetic activation of buffalo oocytes with or without zona pellucida by ethanol or calcium ionomycin has been previously reported (Gasparrini et al. 2004b; Shah et al. 2008; Singh et al. 2012). Furthermore, proteomic analysis of parthenogenetic embryos showed that energy metabolism is dysregulated (Pu et al. 2020), and comprehensive analysis should be performed to improve the developmental potential of parthenogenesis.

Although sperm provides natural stimulation for oocyte activation, oocytes can also be activated parthenogenetically through a variety of physical and chemical stimuli. By treating parthenogenetic activation of oocytes with or without 6-dimethylaminopurine (6-DMAP), various chemicals were used to induce Ca 2+ oscillations and increase Ca 2+ concentration. These chemicals activate oocytes through a sperm-like mechanism by inducing higher levels of intracellular Ca2 +. It has been reported that treatment with iodomycin and 6-DMAP can activate nuclear transfer buffalo embryos of intact somatic cells (Shi et al., 2007) and manually cloned embryos without zona (George et al. 2011).

In a study conducted by Singh et al., (Singh et al. 2021), the expression profiles of CX37, CX47, GDF9, BMP15, ZP2, ZP3, GLUT1, HSF1, ZAR1, BAX, and MCL1 were studied, and the expression profiles of buffalo embryos produced by parthenogenetic. Studied at different stages, indicating that the dynamics of the above genes play a role in early embryonic development.

7.9 Somatic Cell Nuclear Transfer

Cloning is an advanced technology for producing embryos, an exact gene copy, or a copy of the animal you want to reproduce. This can be done via somatic cell nuclear transfer (SCNT) or embryonic division. This technology offers the ability to replicate a copy of the super buffalo, which produces large quantities of milk or meat, which will maximize and improve the profitable buffalo production system. This means fewer animals are needed to produce the required amount of milk and meat, less labor, water, and feed resources, less area is required, and less methane gas is secreted, which is good for the environment. In India, successful SCNT application has resulted in the production of superior buffalo bull using donor cells from tail-skin biopsy and seminal plasma (Selokar et al. 2019). Through cloning, farmers can choose the best animals with the best genetics and copy them into equally strong and healthy animals. The potential benefits provided by this technology in animal husbandry are enormous, but its implementation efficiency in water buffalo is slow and challenging.

According to reports, using fetal fibroblasts or granules as donor cells, buffalo were successfully cloned and buffalo were successfully cloned (Shi et al. 2007), The nucleus of river buffalo ear fibroblasts was fused into the cytoplasm of marsh buffalo oocytes (Yang et al. 2010), adult skin fibroblasts as nuclear donors, cloned vitrified embryos without zona pellucida were obtained from enucleated oocytes.

7.10 Intracytoplasmic Sperm Injection

In 1992, intracytoplasmic sperm injection (ICSI) was introduced into clinical practice to overcome male infertility and the failure of conventional IVF cases. Today, ICSI has been used in most IVF cases around the world and can be used for almost all signs of infertility and its pregnancy rate has always been high.

Recent studies have shown that if the morphological characteristics of the sperm selected for microinjection are examined at very high magnification, ICSI results can be further improved. This led to the introduction of IMSI, which offers the advantage of using the morphologically most appropriate sperm for ICSI.

ICSI-derived blastocysts were obtained by microdroplet vitrification of mature buffalo oocytes in vitro (Liang et al. 2011). Although recent progress has been made in establishing and improving the method of cryopreservation of swamp buffalo oocytes, the developmental capacity of vitrified oocytes in this species is still very low (Attanasio et al. 2010). Indeed, the most effective cooling method and CPA treatment protocol for cryopreservation of oocytes of this species have not yet been determined. Cryopreservation is known to cause changes in the zona pellucida (ZP), leading to decreased fertility (Carroll et al. 1990). However, these structural changes can be overcome with ICSI (Carroll et al. 1990; Porcu et al. 1997).

7.11 Proteomics

Proteomics is the analysis of the entire protein complement of a specific group of specific cells, tissues, or organisms under specific and specific conditions. At present, it depends on decades of technological and instrumental development. Proteomics can provide us with direct information on the molecular mechanisms that drive embryonic development because proteins are the executors of most development plans. Furthermore, proteomic analysis was performed to understand the developmental mechanisms of oocytes and embryos in various species, including buffalo (Chen et al. 2018; Fu et al. 2016), cows (Memili et al. 2007; Peddinti et al. 2010), and pigs (Kim et al. 2011; Powell et al. 2010).

These developments include advances in mass spectrometry (MS), protein fractionation technology, and bioinformatics. The mechanism that regulates embryonic development under reduced oxygen pressure remains elusive. Therefore, Shahzad et al. (2020c) cultured buffalo embryos with less than 5% or 20% oxygen and used iTRAQ-based quantitative proteomics. Functional analysis showed that 43 differentially expressed proteins are related to glycolysis and the degradation of fatty acids. The data show that in embryos grown under hypoxic conditions, greater lipid degradation, higher cholesterol levels, and a higher ratio of unsaturated fatty acids to saturated fatty acids may be related to improved cryogenic survival. Various approaches can be explored through proteomics, such as the proteomic profile of uterine fluid and the highly fertile buffalo oviduct.

7.12 Transcriptomics

A set of transcripts or transcriptomes can help decode information on metabolism and cell function (Wang et al. 2009). The transcriptome is a complete collection of transcripts in a cell, which can be generated using hybridization (microarray) and next-generation RNA sequencing (RNA-seq).

Previously, in other non-ruminant animal species, such as Siberian hamsters, there have been reports of the relationship between changes in mRNA abundance and changes in the photoperiod of specific genes involved in follicle formation in the ovary (Salomon et al. 2018). They further investigated seasonal changes in transcriptome and miRNA expression in another short-day breeding sheep and observed a decline in oocyte capacity during NBS, as indicated by impaired embryonic development in vitro (Mara et al. 2014).

Furthermore, it has been reported that transcriptome variation in sheep ovaries may be related to the breeding season (Chen et al. 2012). In a study conducted, it was documented that the season significantly affects female fertility in buffaloes and the oocyte transcriptome of genes related to follicle formation and the acquisition of oocyte abilities (Capra et al. 2020).

As mentioned above, the latest molecular tools are being used to study the development of buffalo embryos. However, research efforts should be strengthened to accelerate buffalo genetic improvement. Recently, the world is using genome engineering technology to achieve desired traits in the next generation. Genetic engineering techniques, such as using CRISPR CAS-9 for gene editing, have broad prospects. Therefore, scientists engaged in water buffalo improvement work should also pay attention to developing buffalo genetic engineering programs.

7.13 Conclusion

Obtaining more specific information on the requirements of buffalo oocytes and embryos in vitro has led to a significant increase in the efficiency of IVEP in this species. However, despite the sharp increase in blastocyst production, the pregnancy rate is still very low and after the transfer of cryopreserved embryos from live animals, only a few calves have been produced. In addition to the small number of oocytes recovered (which is an intrinsic characteristic of the species), an important limiting factor is a low resistance to cryopreservation of embryos cultured in vitro from buffalo, which can also be considered due to poor culture conditions and a higher cytoplasmic lipid content.

In future perspectives, the first limitation can be partially overcome by selecting donors based on their potential for follicular formation, and the first limitation can be partially overcome by optimizing the stimulation regimen to promote follicular growth and thus the recovery of oocytes. In addition, to improve the cryopreservation efficiency of embryos of this species, further research is needed to optimize the in vitro culture system and cryopreservation procedures, paying particular attention to the minimal volume vitrification method, which has produced results. Promising in vitro. Improved freezing of buffalo IVEP embryos will lead to advanced breeding strategies and become a routine procedure for buffalo farming.

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