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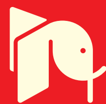
New Insights into Theriogenology

Edited by Rita Payan-Carreira



NEW INSIGHTS INTO THERIOGENOLOGY

Edited by **Rita Payan-Carreira**

New Insights into Theriogenology
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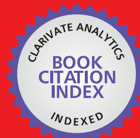

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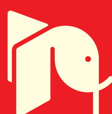

IntechOpen Book Series
Veterinary Medicine and Science
Volume 1

Rita Payan-Carreira earned her veterinary degree from the Faculty of Veterinary Medicine in Lisbon, Portugal, in 1985. For some years, she was a veterinary practitioner and teacher. She worked at the University of Trás-os-Montes and Alto Douro for more than 30 years, where she taught in the Integrated Master in Veterinary Medicine (Animal Reproduction, Gynecology and Obstetrics) field, in the Zootechnics Engineering Master, and also in the Ph.D. courses in Animal Science and in Veterinary Sciences. She is now a full professor at the University of Évora, in the department of Veterinary Medicine. Rita has participated in several joint academic activities with foreign universities, and she is part of several international networks on veterinary theriogenology. She is currently the President of the Portuguese Society for Animal Reproduction. Her primary research areas include the molecular markers of the endometrial cycle and fertility and the embryo–maternal interaction. Related to her teaching, Rita's interests focus on the active learning strategies to develop critical thinking skills, particularly those fostering clinical reasoning and medical decision making as well as those related to the development of interpersonal and inter-professional communication skills. Often, she supervises students preparing their master or doctoral thesis. She is also a frequent referee for diverse journals in the area. For further information on her scientific publications, please visit http://orcid.org/0000-0001-5225-4510.

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Scope of the Series

Paralleling similar advances in the medical field, astounding advances occurred in the Veterinary Medicine and Science in recent decades, fostering a better support to animal health and more humane animal production, a better understanding of the physiology of endangered species, to improve the assisted reproductive technologies or the pathogenesis of certain diseases, where animals can be used as models for human diseases (like cancer, degenerative diseases or fertility), and even as a guarantee of public health. Bridging the Human, Animal and Environmental health, the holistic and integrative "One Health" concept concept intimately associates the developments within those fields, projecting its advancements into practice.

This series aims to tackle different fields in the animal-related medicine and sciences, providing thematic volumes high quality and significance in the field, direct for researchers and postgraduates, giving us a glimpse into the new accomplishments in the Veterinary Medicine and Science field. Addressing hot topics in veterinary sciences, we aim to gather authoritative texts within each issue of this series, providing in-depth overviews and analysis for graduates, academics and practitioners foreseeing a deeper understanding of the subject. Forthcoming texts, written and edited by experienced researchers from both industry and academia, will also discuss scientific challenges faced today in Veterinary Medicine and Science. In brief, we hope that books in this series will provide accessible references for those interested or working in this field and encourage learning in a range of different topics.

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Preface

It is with my great pleasure that I present you with this book on the theriogenology field. Albeit that this name is still not widely appreciated, it reports to the knowledge of all the aspects of animal reproduction (physiology, pathology, clinics), including veterinary obstetrics, veterinary gynaecology, veterinary andrology and also the assisted reproduction biotechnologies. This variety of topics is reflected in the content of the current book.

Under the title of *New Insights into Theriogenology*, this book contains several papers tackling some particular translational aspects of animal reproduction and reproductive medicine. With evolving technology and a continual increase in knowledge, regarding domestic pets or agricultural animals, new information is available on diverse, interesting topics in this broad field.

The content of *New Insights into Theriogenology* reflects the individual experience of the authors, who developed a number of themes identified as essential or interesting in the field. As it is, new opportunities were opened for productive collaborations between the disciplines of theriogenology and authors worldwide. As a group, the editor and the authors, supported by the publisher, intend to share with you our expertise, experience and understanding of multiple relevant aspects that are addressed in the field of theriogenology, sometimes reflecting our personal beliefs, always supported by relevant available information. We tried to provide you with current, specialised information that may be useful to students, clinicians and researchers. All the credits to the book content should be given to the authors, and I am thankful for their collaboration, and the enthusiasm shared in this joint adventure, and the confidence they put in me for coordinating their efforts.

As the proposed themes emerged, were debated and revised, they were gradually shaped into two main sections. In Section 1, several chapters cover the molecular and cellular aspects related to the reproductive physiology – hence its title "Current Topics in Reproductive Physiology". Under this label, the first chapter by Dr. Perumal addresses the main concerns regarding the Mithun female reproduction, a species in danger of extinction living in the Himalayan foothills of South/Southeast Asia. In the second chapter, Dr. Sebastiano tackles the importance of spermatogenesis in male fertility and introduces some new experimental techniques (e.g., spermatogonial stem cells, or stem cell therapy) to revert infertility. Dr. Rak and colleagues, in the third chapter, review the actions of adipokines in the regulation of ovarian function and compare this regulatory system in three different species: the cow, the pig and the chicken. This section is closed with the chapter by Dr. Baykalir and colleagues, discussing the use of some proteome analysis techniques available in animal reproduction.

Section 2 addresses "Hot Topics in Reproductive Medicine" in which the chapters on assisted reproductive techniques were presented. It starts with the chapter by Dr. Quintela Aries

and collaborators, on subclinical endometritis in the cow, which remains a critical cause for poor fertility in the postpartum dairy cow. The role of apoptosis in the early embryo loss in dairy cows, another significant concern in veterinary gynaecology, is addressed in the following chapter, by the team lead by Dr. Moreira da Silva. The next chapter, by Dr. Garcia, reviews the embryo manipulation techniques in rabbits, and its usefulness in translational medicine and embryology studies. Also in the field of assisted reproduction, in the last chapter in this book, Dr. Sato presents the intraoviductal instillation of a solution to transfect preimplantation mammalian embryos in vivo.

I would like to leave also a word of appreciation to the Intech Author Service Manager, Ms. Marijana Francetic, for her help during the revision and production stages of this book, as well as to all other InTech technicians that provided the best of their knowledge during all the production phases leading to the final product.

We hope that this book inspires you to embrace these themes, foster the debate on particular topics and may be used as a start-up source for exploring the theriogenology field.

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Section 1

Current Topics in Reproductive Physiology

Chapter 1

Reproduction in the Female Mithun

Perumal Ponraj

Additional information is available at the end of the chapter

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Abstract

Mithun is a domesticated beef bovine species in Himalayan foothills of Southeast Asia. It inhabits at an altitude from 300 to 3000 m MSL, often under extensive grazing system. Mithun is a very fertile animal, able to produce one calf/year. Mithun can interbreed with other bovine species, but male offspring are sterile. This chapter intent is to gather and discuss available information on several aspects of reproduction in female Mithun. The morphology of the reproductive organs is different from cattle and has a longer reproductive tract and pregnancies compared to cattle. Although its estrus period is longer than in cow, Mithun usually displays silent heats, requiring a breeding bull for heat detection. Seasonal fluctuations on reproductive pattern have been reported, which are related to forage availability and quality. Calving in summer takes longer to resumption of cyclic ovarian activity than calving in winter. As Mithun is an important socio-cultural-religious-economic asset in India, to counter some short comings, new breeding strategies have been introduced like multiple ovulation and embryo transfer technology. A good understanding of different aspects of reproduction is crucial to support effective reproductive managements to enhance socio-economic status and cultural importance of tribals to preserve Mithun germplasm to be used in future.

Keywords: reproductive traits, reproductive problems, biometry of the female genitalia, reproductive physiology, assisted reproduction

1. Introduction

Mithun is a unique domesticated bovine species raised in the Himalayan foothills of South/Southeast Asia. In India, they can be found in the North Eastern hilly regions, such as Arunachal Pradesh, Manipur, Mizoram, and Nagaland. It is commonly believed that Mithun originated more than 8000 years ago, from the wild Indian Gaur, *Bos gaurus* [1, 2]. The spelling

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word such as Mithun (exact in pronunciation) is commonly practiced for the hilly bovine species (*Bos frontalis*); however, "Mithan" is also a common pronunciation and spelling while in some other cases, "Mythun" is another term used. Mithun are also known as "Gayal" in the Indian subcontinent. In Myanmar and Bhutan, it is called Mithan, whereas in China it is called Dulong cattle. This name probably originated from Assamese dialects.

The massive unique beautiful animal is anatomically and physiologically well adapted to altitudes ranging from 300 to 3000 mMSL. Mithun is a social animal; they establish small groups, usually containing one adult male and several females and juveniles. The multiple purpose use of Mithun is well acknowledged. It is a potential source of delicious meat [3], and it can also be used for the improvement of local, native cattle breeds. Mithun is a recent addition to the field of scientific animal husbandry. In the future, foreign exchange could be earned by exporting Mithun's products, namely meat, milk, hide, or skin. The Gayal or Mithun is larger and heavier compared with native domestic cattle breeds. Mithun hide has almost three times the size and thickness that of native cattle [4].

Human involvement and the manipulation of biodiversity severely affected Mithun′ habitats and increase the pressure that drove them deep into the jungle, in areas farther into the border [5]. Even though Mithun is not yet considered an endangered species, it has been subjected to severe noncyclic population fluctuations on a local or national or regional basis. Mithun population is decreasing gradually, mainly due to the local unavailability of certified breeding bulls, the increase of inbreeding practices, the decline in the grazing land, and also because a suitable breeding and feeding management is still to be established in Mithun rearing areas.

A good understanding of the reproductive processes in Mithun cows and its interaction with the environmental factors is crucial to implement effective reproductive management measures, to preserve its population, and to enhance the socio-economic status and the cultural importance of Mithun in the local tribal populations. Hence, a holistic approach from all areas of animal husbandry and veterinary programs will allow to exploit the production potentiality of Mithun. The present review aims to bring the different aspects reproductive physiology of Mithun in concise manner for the benefit of Mithun farmers, breeders and technicians.

2. Taxonomical classification of Mithun

Based on the available literature, the taxonomy of Mithun is presented below [6]:

- Kingdom: Animalia
- Phylum: Chordate
- Sub-phylum: Vertebrata
- Class: Mammalia (mammals)

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- Sub-class: Eutheria (placental mammals)
- Order: Ungulate (hoofed mammals)
- Sub-order: Artiodactyla (even-toed animals)
- Pecora/Ruminantia (true ruminants)
- Family: Bovidae (hollow-horned animals)
- Sub-family: Bovinae
- Genus: *Bos*
- Species: *frontalis*
- Scientific Name: *Bos frontalis*

3. General description

There is no clear-cut historical record on the origin and domestication of Mithun. European studies documented, for the first time, the existence of this animal during the nineteenth century. The history of domestication of this animal has been reconstructed based on the native people' traditions and present daily practices. Native traditions suggest that Mithun is an early domesticated form, descending of related wild animals that were attracted to human beings by its craving for salt. Simultaneously, the human motives focused on obtaining an animal to sacrifice for meat. Three different hypotheses have been proposed on the origin of this mountain animal: (1) Mithun is the domesticated form of the wild Gaur [1, 2, 6, 7]; (2) Mithun is a hybrid of bull Gaur with a zebu cow [8] or either a *B. indicus* or *B. taurus* cow [9]; or (3) Mithun is a descendant of a wild Indian bovine now extinct.

As the male originating from a cross between Mithun and cattle is usually sterile, the second hypothesis can be discarded, since there is little chance for Mithun to be a stable hybrid of Gaur and cattle. Hybridization practices with domestic cattle, however, make it more complicated to understand the origins of domestication and Mithun identification. The third hypothesis is questionable as there is little similarity in the appearance of pure Mithun and common cattle available in Mithun-inhabited areas. Conversely, the physical features of Mithun and Gaur are similar and it is difficult, even for an expert, to differentiate whenever Mithun and Gaur are kept together. Moreover, no sterility barrier exists between Mithun and Gaur, and both animals possess 29 pair of chromosomes. Due to their resemblances, some animal taxonomists had once mistaken the Mithun as a type of domesticated Indian Gaur [10]. The fact is that many morphological, physiological, and reproductive parameters are different in Mithun compared with cattle, strongly support the hypothesis that the Mithun is a domesticated form of wild Gaur; whose domestication occurred some 8000 years ago. Even according to the latest taxonomical classification, both Mithun and Gaur are classified under the same species *Bos* [1, 2, 7]. Two different "breeds" of *B. frontalis* have been reported in the northeastern region of India [11].

The divergence time estimated for *B. gaurus* and *B. frontalis* provide further support in favor of the hypothesis that assumes its origin from an extinct wild progenitor.

Result from genetic studies display contradictory information on the origins of Mithun, which could be related to the existence of different genetic subpopulations that are originated by the habitat fragmentation, which would allow the breed to present different genetics adrift. Baig et al. [12] reported the occurrence of two haplotypes of *B. indicus* and one of *B. taurus* in *B. frontalis*, reinforcing the gene flow occurring between the domestic cattle and *B. frontalis* population. This genetic introgression of domestic cattle poses a severe threat to the diversity of *B. frontalis* in their respective regions [10, 13]. Nevertheless, Shan et al. [14] speculated that Mithun was neither a domesticated type of Gaur nor a descendant of Gaur and cattle crosses; whereas, Lan et al. [15] reported that the restriction type of mtDNA of Mithun was the same as that of zebu, so the maternal origin of Mithun had a close relationship with zebu while Y chromosome of Mithun was the same with Gaur. They speculated that Mithun might be the descendant of male gaur and female zebu. Based on the partial sequences of cytochrome *b* (*Cyt b*) gene, Ma et al. [10] reported that the Mithun was not a crossbreed descendant, but an independent species of *Bos* from *Bos indicus*, *Bos taurus*, and *Bos gaurus*. Li et al. [13] reported based on the complete *Cyt b* gene sequences that Mithun could be grouped into three embranchments: one embranchment clustering with *Bos gaurus*, another clustering with *Bos taurus*, and the third clustering with *Bos indicus*. Phylogenetic analysis indicated that Mithun might be the Gaur in the domesticated form and also that a significant proportion of the Mithun bloodlines in China were mixed with other related species of bovine [16]. Based on the mtDNA and *SRY* gene sequences, Gou et al. [17] reported that the Mithun from the Yunnan province originated from the hybridization between a male *Bos frontalis* and female *Bos taurus* or *Bos indicus.* Tanaka et al. [18] reported based on the mitochondrial *Cytb* gene that the ultimate maternal origin of Mithun was Gaur and indicated that it was directly domesticated from Gaur. Baig et al. [12] argued that the *B. frontalis* should be treated as a separate species/sub-species based on the mitochondrial DNA (mtDNA) analysis, and not merely as a domestic form of *B. gaurus.*

4. Distribution of Mithun

Mithun has a limited geographical distribution. It is mainly found in the tropical rainforest of North-eastern hill States of India (Arunachal Pradesh, Nagaland, Manipur, and Mizoram), with also small numbers of animals in Myanmar, Bangladesh, China, and Bhutan. However, as per the nineteenth Livestock Census of India [19], the total population of Mithun in Indian sub-continent was 297,289 (**Table 1**).

In the moist forests of the northeastern Himalayan foothills, Mithuns are free-ranging animals; as they do not have an established breeding plan, they are sometimes described as semi-domestic animals [20]. The Naga people encourage interbreeding between the wild Gaur bull and their Mithun cows and regard this practice as an improvement on the breed [20, 21]. In east Bhutan and Arunachal Pradesh, Mithuns are particularly prized for sire the local cows (*Bos indicus* type) [20, 22]. In Bhutan, particularly in the eastern regions, the farmers are crossing the Mithun bulls with local Siri females, as the female crossbred offspring present higher milk yields, the milk

Indian states	1997	2003	2007	2012
Arunachal Pradesh	124,194	184,343	218,931	249,000
Nagaland	33,445	40,452	33,385	34,871
Manipur	16,660	19,737	10,024	10,131
Mizoram	2594	1783	1939	3287
Total	176,893	246,315	264,279	297,289

Table 1. Trends in Mithun population in North-East India.

is being used mainly for butter and cheese. The Arunachal Pradesh tribes also use Mithun for breeding of local cows. The F_1 hybrids produced, named as Jatsha/Jatsa (male), are very strong and docile and are used for plowing, whereas the females, named as Jatsham/Jatsamin, yield more milk than purebred Mithun or local cows. Jatsham is a valuable dairy stock for these people [20, 22], but Jatsha have fertility problems. Because of male infertility, the Bhutanese farmers use to backcross the female hybrids to domestic cattle bulls for four generations [23]. In the F_2 generation (male Mithun × F_1 female), the males, called as Nupsa, are used for plowing; whereas the females, called as Nupsamin, are reared for the increased milk production [24].

Mithun is the only wild relative of zebu cattle found in the hilly regions of Chittagong in Bangladesh, especially in the Bandarban district. Some tribal families raise Mithun with native cattle and their cross natural offspring—called "Tang gaur"—can be found in the local markets. In Bangladesh, these animals are mainly used as a high-price sacrificial animal during religious festival [25]. In India, the Mithun female cross is called Phre [26]. In the eastern Himalayas, Mithuns are bred with Dzo (the product of a cross between a yak and a cow) or with yaks. Those crosses evidence the usual hybrid fertility/sterility pattern and therefore are used mainly for traction and milk production (males and females, respectively) [24].

5. Biometry of the genitalia of Mithun cow

The reproductive tract of the Mithun cow is longer than the cow's and although lying in the pelvic cavity, it hangs forward into the abdominal cavity. The vulva, the external opening of the urogenital tract, is smaller compared to the cow. The vulvar cleft (the ventral slip between the two labia) nests the clitoris, also less developed than in cattle [27, 28]. The segment showing the most important changes in size is the uterine horns, which is influenced not only by age, but also by parity and the time length elapsed from the last calving [27]. They are usually about 20 cm long in a virgin heifer, but may reach up to 40 cm long in older cows. The uterine horns curve forward, downward, outward, and then upward like the curled horns of a ram. As in cows, the uterine horns are quite firm and erect during estrus, and the intercornual space is very distinct. Regarding the internal morphology of the uterus, about 125–150 (125.17 ± 18.52) caruncles can be observed as small, raised, button-like areas [27]. The ovary is oval-shaped and varied between 1 and 2 cm in diameter. A corpus luteum with a distinct neck is palpable in the ovary between days 8–14 of the cycle; thereafter, neck becomes indistinct

Segment of the genital tract	Measurements			Notes
	Length (cm)	Thickness (cm)	Width (cm)	
Vulva	14.33 ± 3.25 (ventral) 12.50 ± 3.73 (dorsal)	0.20 ± 0.01		
Vagina	21.83 ± 2.16	0.40 ± 0.06		
Cervix	6.00 ± 0.35	2.16 ± 0.18	1.08 ± 0.11	3.17 ± 0.37 annular rings
Uterine body	5.45 ± 0.70	0.49 ± 0.05	1.98 ± 0.41	
Left uterine horn	19.0 ± 2.0	0.43 ± 0.09	0.97 ± 0.32	
Right uterine horn	21.16 ± 2.11	0.47 ± 0.09	2.18 ± 0.30	
Left oviduct	33.42 ± 5.63			Diameter 0.30 ± 0.10 cm
Right oviduct	35.17 ± 5.90			Diameter 0.29 ± 0.09 cm
Left ovary	2.75 ± 0.15	1.58 ± 0.44	1.67 ± 0.42	Weight 4.44 ± 1.20 g
Right ovary	3.02 ± 0.29	1.68 ± 0.45	1.73 ± 0.24	Weight 3.82 ± 0.59 g

Table 2. Biometry of the female genital tract in Mithun cows (adapted from [27]).

and at about 21 days of the cycle only a scar of the CL is present [28]. The available data on the reproductive tract biometry is provided in **Table 2**.

6. Endocrinology of the estrous cycle

As it occurs in the cow, the progesterone (P4) concentration is the lowest at estrus and gradually increases until day 6 of cycle (day 0 represents the ovulation day) [29]. From day 7 onward, the concentration of progesterone raises sharply to reach a peak on day 11. After this day, progesterone concentrations decrease steadily to reach a minimum at the following day of estrus. Secretary pattern of FSH and LH has shown significant negative relationship with P4 during first and last 6 days of the cycle. A biphasic peak has been reported for both FSH and LH in Mithun cyclic females. Concentration of FSH and LH starts to increase from day 5 and day 6 before estrus, respectively. Then they attain their first peak on day 2–3 before estrus, and thereafter, their levels decrease, and the second peak is observed on the day of estrus. The concentrations of both gonadotropins decrease thereafter to basal level, those of FSH (on day 3 or 4 of the cycle) 1 or 2 days earlier than those of LH (on day 4 or 5 of the cycle) [29].

Dhali et al. [29] reported the existence of two kinds of patterns regarding the estrogen secretion through the estrous cycle of Mithun females. Some cows displayed only one peak of estradiol 17-β (18.92 ± 3.76 pg/ml) during the entire estrous cycle, on day 4 before estrus. In these females, the FSH peak reported before estrus was significantly lower compared to females

presenting two peaks of estradiol 17-β. The first peak (20.32 ± 3.61 pg/ml) was recorded 4 days before estrus and the second peak (19.23 ± 2.14 pg/ml) on the day of estrus [29].

The mean length reported for the Mithun estrous cycle is 21.2 ± 0.3 days (19–24 days). The emergence of the first follicular wave has been proposed to occur just after ovulation, while the FSH values are still elevated and the progesterone in low values [29]. The emergence of the second follicular wave would occur approximately at day 14 of the cycle, in association with the raising FSH concentrations that will originate the first FSH peak, along with increasing LH and E2 concentrations. The functional capacity of the CL starts decreasing from day 11 onward and circulating P4 concentration starts reducing. This decrease allows the rising of FSH and, around day 16, the selection of the dominant follicle from the second follicular wave. The deviation of ovulatory follicle was proposed to occur around day 18 of the cycle. The circulatory concentration of E2 starts to decrease with the regression of subordinate follicles. During the late stage of cycle, low concentration of P4 stimulates the rise in LH which helps in the final maturation of ovulatory follicle and triggers ovulation [29].

7. Reproductive traits

No breeding programs can be implemented unless the animal has sound reproductive traits. Earlier reports [30, 31] showed that Mithun has a fairly good reproductive performance. Mithun is a polyestrous animal. Females become sexually mature at 2–3 years of age. Mithun bulls become mature at 3–4 years of age. Mithun cows present a high reproductive efficiency, as indicated by the reported calving-related traits (calving interval: 402.85 ± 3.04 days, gestation length: 296.25 ± 0.77 days, birth weight: 20.85 ± 0.24 kg) [32], which permit to obtain one calf in a year. Mithun productive life ranges from 16 to 18 years.

Different studies present slight differences among values respecting the reproductive traits in Mithun compared with local cattle (*Bos indicus*) and buffaloes. **Table 3** summarizes the available information. In general, Mithun requires a higher number of services than indigenous cows when artificial insemination is used, which could be associated to improper heat detection, lack of knowledge of the inseminator about the characteristic morphology of the reproductive organs in Mithun. Also, the concentration of spermatozoa per dose or incorrect insemination may be the cause of more services (artificial insemination) for conception in this animal [34]. The length of pregnancy is in general longer in Mithun than the European cows [4, 36], but it is shorter for Mithun × cattle mating (281.7 ± 1.2 days) [34]. This suggests that purebred Mithun calves needs a little longer to mature than crossbred calves [34].

The average birth weights reported in male and female Mithun calves are presented in **Table 4**. In general, female calves born weighed lighter than the males [40]. These differences are maintained across pregnancies [34] although Haque et al. [39] only found significant effects of parity in the birth weight of female calves.

Seasonal fluctuation in the Mithun reproductive pattern has been described. A higher number of calving (33.3%) occurs during monsoon and autumn seasons followed by 16.7% calving rate was observed during summer and winter seasons [34], reflecting a higher conception rate

Reproductive traits	Mithun	Cattle [33]	Buffalo [33]
Type of estrous cycle	Polyestrous [4, 31, 34]	Polyestrous	Polyestrous
Age at first estrus	598.2 ± 168.4 d [34] 527.9 d [35] 22–39 mo [31]	15 (10–24) mo	21 (15–36) mo
Age at first conception	723 ± 169.9 d [34] 779.70 d [4] 644.8 d [35]		24–36 mo
Weight at first estrus (kg)	247.8 ± 35.1 [25, 34]		250–275
Length of estrous cycle (d)	21.9 ± 2.9 [34] 19–24 [31] 21.65 [4]	21 (14–29)	21 (18–22)
Duration of heat (h)	45.4 ± 12.2 [34] 10–36.9 [31] 42.68 [4]	18 (12–30)	21 (17–24)
Service per conception:	2.30 [4]	1.91	1.3/1.76
Natural breeding	1.4 [34]	1.27	1.15
Artificial Insemination	5.0 [34]	1.40	1.48
Gestation period (d)			
Purebred	296.1 ± 3.9 [34] 282–320 [31] 293.27 [4] (293–303) [36] 296.25 ± 0.77 d [32]	280 (278–293)	315 (305–330)
Mithun × cattle	281.7 ± 1.2 [34] 283 d [35]		
Age at first calving (d)	1014.4 ± 260.3 [34] 1192.50 [4]	30 (24–36) mo *B. indicus*: 44 mo Crossbred: 34 mo	42 (36–56) mo
Postpartum estrous (d)	96.2 ± 24.0 [34]	Uterine involution —45 d 1st Ovulation—30 (10–110)	35 (16–60) 75 (35–180)
Days open (d)	172 ± 83.5 [34] 203 [4]	55–85	35–185
Calving intervals	349–395 d [31] 402.85 ± 3.04 d [32]	13 (12–14) mo	18 (15–21) mo
Natural service	465 ± 80.5 d [34]		

Reproductive traits	Mithun	Cattle [33]	Buffalo [33]
Artificial insemination	838 ± 158.5 d [34]		
Home region	300–400 days [37]		
Ex-situ	553 days [38]		
Optimum time for service after the onset of heat	21–30 h [34]	Middle of estrus	
Lactation length			315 ± 28.28 d
Productive life			14.3 ± 2.46 y
Lifespan			21.0 ± 3.36 y
Lifetime number of calving			10.4 ± 1.30

The sources are referenced within square brackets.
h—hour; d—days; mo—months; y—year.

Table 3. Comparison of Mithun reproductive traits with those of indigenous cattle and buffaloes values are given as average; when available, range values are provided within brackets.

Gender	Average birth weight (Kg)			
	Overall	**At 1st lactation**	**At 2nd lactation**	**At 3rd lactation**
Male calves	24.3 ± 6.9 [39] 21.67 ± 0.15 [32]	19.7 ± 2.1 [34]	21.3 ± 1.5 [34]	23.0 ± 0.0 [34]
Female calves	20.20 ± 4.08 [39] 19.63 ± 0.21 [32]	15.6 ± 1.4 [34]	19.7 ± 2.6 [34]	21.3 ± 1.8 [34]
Nonspecified	19.1 ± 3.3 [35]			

The sources are referenced within square brackets.

Table 4. Birth weight in Mithun calves.

in winter (December–February). This could be explained by the availability of green fodder after summer in the period of monsoon, autumn, and winter which improve the body condition and health status of breeding animals [41]. It was also reported that a decreasing trend exists to a shorter postpartum anestrus in Mithun calving in autumn and monsoon seasons (87.3 and 94.2 days, respectively), compared to those calving in summer or winter (158.7 and 174.7 days, respectively) [34]. A retrospective study on the calving trends in semi-intensive farms showed the highest birth rate in September, December, and January, while the lowest rate was recorded in May and June [40].

On respect to the fertile period, it has been demonstrated that a 70.6% successful conception is obtained when the service (natural mounting) is provided at the middle of estrus, within 21–30 h of heat. If artificial insemination is used, a 100% successful conception can be obtained when the insemination occurs within 31–40 h of heat [34].

Correlations were established between the weights at first calving, pregnancy length, the length of the service period and calving interval for Mithun. Negative correlations were high

for the weight at first calving and the service period (−0.684) or the calving interval (−0.577), but low for the weight at first calving and the pregnancy length (−0.138). Moderate positive correlations were found for the pregnancy length and the service period (0.475) or the calving interval (0.514). A positive high correlation was observed between the service period and calving interval (0.881) [31]. Under extensive free-range rearing systems, a suitable practical method for selective breeding is the introduction of tested and superior bulls, in a proportion of 1 bull to 10 breeding females, together with the elimination of bull with poor performances (both productive and reproductive). Efforts should be made to replace breeding bulls preferably once in 5 years to avoid inbreeding depression. Under the semi-intensive system, the female can be detected in heat to be bred with superior bulls either through natural service or artificial insemination [37].

8. Estrus behavior and signs

The behavioral signs of estrus most frequently displayed by Mithun cows are accepting the mounting by a Mithun bull, standing to be mounted, and congestion of the vaginal mucosa and swelling of the vulva. In contrast to other bovines [42], estrous signs such as mucus discharge, restlessness, tail raising, frequent urination, and loss of appetite are less pronounced in Mithun cows. The length of estrus is longer in primiparous Mithun cows than in multiparous, due to an elongation of the mounting behavior, Flehmen reflex, and restlessness displayed by younger Mithun females. This allows the primiparous animals to be mounted for more times than the multiparous females. The relative incidences of the behavioral manifestations have been described in Mithun females, and provide a good indicator for distinguishing between primary and secondary estrus signs. Sniffing of the vulva by bulls and standing to be mounted by bulls/other herd mates was recorded in 91.30% each. The increase in the frequency of urination was observed in 82.61% of the female in estrus; alike allowing the bull chin resting on the rump. Only 65.22% of Mithun females in estrus displayed restlessness, which was pronounced in 8.70%, but not pronounced in 56.52% of the females. Bellowing was exhibited by 56.52% Mithun female [42], being frequent in 39.13% of the females, but infrequent in 17.39%. The homosexual behavior during estrus is less prominent in Mithun compared to cows. Seeking for the company of other animals (looking anxiously outside) was found in 56.52% of the Mithun females in estrus. Licking of the body of other animals during estrus was observed in 47.83%, but mounting herd mates during estrus was observed in only 26.09% of females. Reduced food intake or loss of appetite was observed in only 8.70% Mithun females in estrus [43].

During estrus, the vaginal mucosa of Mithun females was recorded as reddish pink (pronounced), pink (moderate), and pinkish (slight) in 34.78, 43.48, and 21.74%, respectively. Edema of vulva was recorded in 82.61% of female Mithuns in estrus, of which pronounced edema and slight edema were recorded in 21.74 and 60.87% of estrus females, respectively. Discharge of the vaginal mucus does not always occur spontaneously in Mithun females in estrus, and has only been reported in 78.26% of the females. In 39.13% animals, the mucus was not discharged spontaneously until rectal palpation was carried out. The quantity of genital mucus was scored as copious, scanty, and absent in 34.78, 43.48, and 21.74% of the females, respectively. The

color of the vaginal mucus varied between transparent (61.11%), steel bluish (22.22%), and whitish (16.67%). Also, the consistency of the vaginal mucus varied from thin (55.56%) to thick (44.44%). The occurrence of foam in the mouth was observed in 30.43% animals [44].

9. Pregnancy diagnosis and parturition

For pregnancy diagnosis, the transrectal criteria used in cows also applies in Mithun. The slip of the fetal membranes, distension of the uterine horn, and the presence of CL in the ovary are observed at the earliest by the 6th week of gestation. The pregnant uterus and ovary sink into the abdominal cavity after 3 months of pregnancy, and are not be palpated after 3 months of pregnancy, as described in cattle. The fremitus of the uterine artery is differentiable after 4 months and is much prominent or forceful from 6 months of gestation onwards. Enlargement of the middle uterine artery is observed after 5 months of pregnancy. Pregnancies in the right horn are more frequently observed (60%) in comparison with cattle [45].

The external signs of pregnancy in Mithun are similar to those of cattle, namely the abdominal distension, mammary gland development, and fetal ballottement. The abdominal distension, due to the gradual accumulation of voluminous uterine contents, can be detected after 5 months, but it is prominent only in more advanced stages of pregnancy. The udder development is noticed from 6 months onwards in Mithun heifers, or in the last 1–5 weeks of pregnancy in the case of multiparous cows. The teats are engorged at 12 h prepartum. Edema and relaxation of the vulva, derived from the progressive relaxation of pelvic ligament, is noticeable in the last few weeks of gestation. Three to four days before parturition, the vulvar lips become increasingly droopy and flaccid, along with a marked shrinking of the croup. In 74% of pregnancies, the fetus is balloted or its movement observed through abdominal wall after 6 months of pregnancy [45].

The process of the parturition is more or less similar in Mithun and in cattle. The pregnant mother becomes nervous and tries to leave the rest of the herd just before parturition. They go through the forest area to find a hidden place. They do not come back to the stall and stay in the forest [46]. In Mithun, most of the calving occurs during the night [47]. Signs of impending parturition in Mithun include restlessness, increased micturition at 2–5 min interval and increased walking. Mithun cows give birth in a laying position. **Table 5** summarizes the peri-parturition behavior in Mithun.

Mithun dams start licking the calf immediately after birth. The calf stands up within 22 min of birth, and first sucks colostrum within 30 min of birth [22]. The average birth weight of the calves, irrespective of sex is found to be 21 kg. The calves stood at their feet an average time of 40 min and the average time for first suckling is to be 50 min on day 1 [49]. During this time period, the dam threats any person or animal that closely approaches the baby. She browses and grazes around her newborn baby so that she can keep a sharp watching to her calf. After 1 or 2 days, she usually comes back to her herd. The calf brows and play around her mother, and feeds of milk 12–15 times in a day. At the 1st week, the Mithun mother is very careful about the safety of her offspring. After 1 week, she gradually returns to her normal and the calf was found moving freely in the herd [25, 34].

Parameters	Groups		
	Nagaland	Arunachal Pradesh	Mizoram
Duration of concentrate feeding (min)	200	199	84
Duration of tree leaves feeding (min)	96	458	538
Duration of drinking (min)	1.06	2.02	2.92
Duration of standing time (min)	807	971	936
Frequency of standing (no.)	12	8	11
Duration of lying time (min)	463.2	373	450
Frequency of lying down (min)	12	8	11
Frequency of defecation (no.)	11	8	8
Frequency of urination (no.)	8	6	8
Duration of parturition process (min)	39.38	63	12.05
Standing on its feet by calf after birth (min)	13.18	18	25.12
First suckling by calf after birth (min)	70.27	68	44.9
Voiding of meconium after birth (min)	28.92	56	1189.1
First urination after birth (min)	443.67	531.5	51.18

Table 5. Peri-parturition behavior of Mithun cow and calf [48].

Events of parturition/intervals	Time lapse (h)
Stage I (stage of cervical dilatation)	
Between the onset of labor to exposition of the water bag	156.17 ± 46.71
Stage II (stage of fetal expulsion)	
Between the exposition of the water bag to forelimbs exposure	25.83 ± 8.94
Between the exposition of the forelimbs to exposition of muzzle	12.16 ± 4.54
Between the exposition of the muzzle to exposition of the head	21.75 ± 9.58
Between the exposition of the head to exposition of the shoulder	11.08 ± 4.54
Between the exposition of shoulder and back	0.68 ± 0.39
Duration of the complete expulsion of the fetus	71.52 ± 18.82
Stage II (stage of expulsion of the fetal membranes expulsion)	
Interval between the expulsion the fetus and fetal membranes	199.65 ± 54.75
Total time for completion of the parturition process	427.33 ± 74.48

Table 6. Relative length of the events occurring in the parturition of Mithuns (adapted from [47]).

The process of parturition is divided into three stages as in the cattle: stage I (stage of cervical dilatation), stage II (stage of fetal expulsion), and stage III (stage of the expulsion of the fetal membranes) (**Table 6**).

The fetal membranes of Mithun present an average weight of 2.51 ± 0.51 kg, and are 194.08 ± 24.97 cm in length and 40.67 ± 2.72 cm in width [47]. The average number of cotyledons

in Mithun is 60.08 ± 5.81, distributed as follows: 75.08 ± 7.15 cotyledons in the gravid horn and 32.50 ± 6.68 in the nongravid horn [47].

Dystocia is common cause for perinatal calf mortality in bovine species. In Mithun, the fetuses are generally found in anterior presentation at calving, and dystocia due to fetomaternal disproportion is rare. When due to maternal causes, abnormal misshaped or small pelvic canal leads predispose the cow to dystocia or calving difficulty [50]. The transverse and vertical diameter of the pelvic inlet and the pelvic area are smaller in Mithun than the cattle. Other parameters such as the height between the hip joint and the croup, or the vertical diameter of the pelvic outlet is larger in Mithun compared to indigenous cattle. Besides, Mithun possess a more muscled hind quarter than the indigenous or dairy cattle [51]. Studies on Mithun pelvimetry would be helpful in establishing breeding programs, where a calving easiness could be a useful trait for selection of breeding animals. Also, a complete soundness breeding exam before the female introduction into reproduction would help to reduce the incidence of dystocia, to cull out the females presenting unsuitable pelvis conformation.

10. Infertility problems in Mithun

Problems associated with reproduction have rarely been observed in Mithun. However, cases such as anestrus, metritis, dystocia, placental retention, and placentophagy or postpartum anestrus have been reported in Mithun [26, 51, 52]. Similarly, the most common reproductive disorders in semi-intensive Mithun farm of Bangladesh include metritis (16.7%), irregular heat (25.0%), anestrus (8.3%), repeat breeder syndrome (8.3%), abortion (16.7%), cervicitis (25%), and calf mortality (24%) [38].

11. Concerns on the sustainability of Mithun populations

Due to the use of continuous inbreeding practices, in part because of the habitat fragmentation and the unavailability of certified sires in its dispersal area and the farmer preferences for a few dominant bulls, a gradual decrease in size, production, and reproductive efficiency has been reported in Mithun. The decline in the population size aggravates the inbreeding depression, and further reduces the breed′ reproductive and productive fitness [53, 54], as well as its survivability [54]. When the genetic variation is reduced within a population, its vigor and ability to adapt to environmental changes are also reduced and may compromise the survival of the breed or population. Therefore, urgent action is needed to conserve this precious mountain bovine.

The fragmentation of the Mithun habitat relates with the traditional rearing system, based on free-ranging herds that only allow the grazing of a limited number of animals, in a particular hill pocket, without migration to other locations. Besides, human interactions and manipulation of biodiversity also affect the habitats of Mithuns, forcing them to migrate deeper into the forests, particularly in the further east, toward the border of the country. Deforestation, poaching, and illegal hunting aggravate the reasons for extinction. Due to deforestation and human settlement,

as well as the conversion of the forest land for agricultural and horticultural activities, the total forest area available and its holding capacity for rearing Mithun cattle has been reduced. Mithun prefers high altitude, hilly terrains of high slopes, and dense forest, as it protects against the direct sunlight and high rainfall, where plenty of fodder trees and shrubs are available. But these areas are reducing due to global warming or climatic changes, which decreases the rainfall and consequently the amount of vegetation or fodder to be used by Mithuns.

Another factor that is contributing to the reduction in Mithun numbers is the incidence of infectious diseases, like tuberculosis, para-tuberculosis, brucellosis, foot and mouth disease (FMD), infectious bovine rhinotracheitis (IBR), and bovine viral diarrhea [55]. The Mithun habitat itself has an important role in the persistence of zoonotic and non-zoonotic diseases within the area. Still, it has been suggested that Mithun in home tract may be more resistant to diseases, compared with *ex-situ* animals [32].

Mithun is grazing with local cattle in the common forest area which has increased the percentage of crossbreeding of Mithun with local zebu cattle [26]. This fact also results in the loss of unique species characteristics of Mithun and may also reflect the failure to correctly respond to behavioral disorders associated with Mithun′ confinement and captivity. These are important drawbacks in the preservation of Mithun. Recently, regular mass Mithun slaughtering for meat purpose has further threatened the population density and the size of this population in the near future. Combining health/breeding risks with the possibility of excessive export for slaughter and the natural habitat destruction, the regime’s commercialization schemes may endanger the Mithuns rather than increase their numbers, and therefore need to be monitored on the name of the species conservation. The mass slaughter of Mithun specimens of excellent quality, mature, characteristic, and big specimens during ceremonies or festival led to loss of good quality germplasm of Mithun, and leaving behind most poor quality or uncharacteristic specimens to be used for a breeding purpose which will impoverish the genetic quality of Mithun progeny [56].

Therefore, considerable and significant efforts need to be taken from all the stakeholders’ quarters, including government, policy makers, agriculture and veterinary departments, researchers, breeders, nongovernment organizations, research institutions and universities, Mithun farmers to prepare proper planning or program to conserve the Mithun in its original form in their habitat.

12. Breeding policy for Mithun

To design and implement a sound breeding policy to improve the production performance of Mithun, a free-range animal, constitutes a real challenge. Indeed, farmers rear this animal under semi-intensive/extensive free range grazing conditions in its natural habitat. The Mithun herds move around the jungle throughout the year and bred naturally with the herd bulls. Under such managemental practices (with low input to maximize the gains) the Mithun improvement for meat and milk purposes through artificial insemination is not an easy task. In this production system, the farmers are not able to monitor their animals for the onset of estrus and take help of superior bulls at their own choice for breeding. Hence, a

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practical approach toward Mithun breeding for better production is probably the introduction of superior male into the herd/farms.

With this purpose, in each state of the northeastern hilly regions (NEHR) recording Mithun populations, Mithun breeding farms should be established, where superior males and females could be maintained under good managemental condition. Separate intensive selection for meat and milk purposes should be the prime objective of these breeding farms. All the essential parameters regarding this rare genetic resource must be taken into consideration at the time of selection for propagation, preservation, and conservation of this valuable species. Simultaneously, superior males generated out of the nuclear stock should be provided to the farmers at the female:male ratio of 10:1. The state Mithun breeding farms will guarantee a continuous supply of superior males, separately selected for meat or milk, according to the farmer's choice. The introduction of artificial insemination using semen collected from superior bull may be an alternative to the improvement of Mithuns reared under intensive systems, in Mithun breeding farms, but it needs to be standardized to explore the possibility for field application. This would foster the development and application of diverse techniques of assisted reproduction that will be revised in the following sections.

13. Biotechnology development in Mithun reproduction

13.1. Standardization of semen preservation and artificial insemination in Mithun

The improvement of the reproductive and productive performance of Mithun demands the females to be breed with semen of superior genetic bulls. This can be easily achieved through the artificial insemination (AI) with preserved semen. Different methods of semen collection and preservation were standardized for the Mithun. Mithun semen was successfully preserved at 4°C (liquid refrigerated semen), for approximately 2 days. It was also successfully cryopreserved in liquid nitrogen using a Tris-egg yolk-glycerol or citrate-egg yolk-glycerol extender with 5% glycerol concentration. Sperm quality was improved (approximately 23–25% increase in progressive motility and count of live sperm with intact acrosome) by adding 5% glycerol in split doses instead of in a single dose. It was also observed that Tris-egg yolk-glycerol extender was better than citrate-egg yolk-glycerol's for cryopreservation of Mithun semen. Inseminations with both frozen and liquid semen successfully produced calves through AI both at farm and field levels [56].

13.2. Estrus synchronization

Protocols for estrus synchronization and timed AI has been developed for the Mithun, including those using prostaglandin F2α (PGF2α) alone, the Ovsynch protocol (GnRH-PGF2α-GnRH), and progesterone-based controlled intra-vaginal drug releasing device (CIDR) [56].

In the synchronization protocol based on prostaglandins, two injections of PGF2α11-day apart are given to cyclic Mithun cows after confirming the existence of a mature corpus luteum. Estrus signs following the injection of PGF2α and indicate the Mithun cows responded to this treatment and insemination can be done. The time from onset of estrus to ovulation is

27.7 ± 0.61 h, with a range of 26–31 h, in PGF2α-treated compared with 26.9 ± 0.31 h, ranging from 26–29 h in untreated cows [56].

The Ovsynch protocol was proved useful for estrus synchronization of cyclic Mithun cows irrespective of the day of the estrous cycle at the beginning of the treatment. This protocol had a respond very good response from Mithun cows; a 75% conception rate was obtained when using this protocol [56].

CIDR is a very useful approach to synchronization of estrus in cyclic as well as postpartum anestrus Mithun cows. It results in more prominent physical as well as behavioral signs of estrus in either cyclic or anoestrous animals compared with nontreated cows in heat. Very interestingly, the use of CIDR at 45–50 days postpartum (dpp) induced estrus at 53–58 dpp, when the uterine involution was completed. In natural conditions, Mithun cows exhibit the first postpartum estrus at around 102 ± 19.6 days postpartum. CIDR is therefore useful and advantageous for the expression of clear physical and behavioral signs of heat, which facilitates heat detection, as well as a higher productive lifespan of at least 50 days [56].

13.3. Multiple ovulation and embryo transfer (MOET)

Mithun is an important livestock species in NEH region of India. The use of artificial insemination programs permits half improvement in genetic makeup through introduction of superior germplasm. However, embryo transfer technology (ETT) may be a useful method for a rapid improvement and multiplication of superior quality germplasm in animals of any species. The technique helps the breeders to produce genetically superior animals within a short period. MOET is not only to be used in genetic improvement of Mithun, i.e., *in situ* conservation, but also used in *ex situ* conservation of Mithun. MOET method can prevent extinction of Mithun in near future [56].

The superovulation/multiple ovulation and embryo transfer technology (ETT) has been successfully standardized for the Mithun species. The first Mithun calf, BHARAT, obtained through multiple ovulation and embryo transfer technology born in March 27, 2012, and the second calf (PRITHVI), born on May 11, 2012, at ICAR-National Research Centre on Mithun (Medziphema, Nagaland, India). Cryopreservation of Mithun embryos has also been standardized. MOHAN, the first Mithun calf, was born in May 12, 2012 from transfer of a 100-day-old cryopreserved embryo, also at ICAR-National Research Centre on Mithun. The standardization of the embryo transfer protocol in Mithun will help in the conservation and propagation of quality germplasm in all the Mithun-inhabited areas of NEH region [56].

14. Conclusion

Mithun is a meat (mainly) animal of Himalayan foothills of Southeast Asia. It can survive in varying environmental conditions and converts crop residue into high quality protein meat. Mithun is a pride and prestigious member of the tribal community. Mithun is a social, friendly, and intelligent animal. Mithun is a very fertile animal and can produce one calf per year. However, its population is fluctuating due to many reasons, although it not yet endangered. In modern

biotechnology era, we need to protect and conserve its germplasm in Mithun home region. Therefore, all the quarters from government to Mithun growers need to take appropriate policy, decision, and action to preserve the Mithun. In ICAR-NRC on Mithun, India, the work of conservation of Mithun in semi-intensive system has been started in last century and successfully implemented in the institute level. Now the implementation was successful in field level by adaptation of the latest nutrition, reproductive, productive, and health management program such as in other bovine and bubaline species. In the reproduction side, artificial insemination, estrus synchronization coupled with timed AI, and embryo transfer technology will help to go a long way to achieve the target of propagating quality germplasm in the farmer's field.

Conflict of interest

The author declares that there is no conflict of interest involved in the present work.

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Chapter 2

Spermatogenesis and Its Significance in Reproductive Medicine

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Additional information is available at the end of the chapter

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Abstract

As infertility rates across nations become a growing concern, the interest in the development of treatments, such as *in vitro* gametogenesis (IVG), increases. This is especially the case for male infertility. For instance, the average sperm count continues to decline across nations, while more adult and pediatric patients survive cancer only to be left with little to no options for fertility restorative therapies. Understanding the male reproductive system and the process of spermatogenesis, however, has proven to be a difficult task. Progress occurs slowly and inconsistencies remain in the literature while reports attempt to better understand spermatogonial stem cells (SSCs) in conjunction with spermatogenesis. Interestingly, stem cell behavior, the decision to self-renewal or commit to differentiation, has shown to be closely linked to the stem cell's microenvironment (i.e. niche). Perhaps the missing pieces required to better understanding spermatogenesis are found in the re-defined perspective of SSC niche dynamics.

Keywords: spermatogenesis, spermatogonial stem cells, stem cell therapy, *in vitro* gametogenesis, organoid engineering

1. Introduction

The Center for Disease Control (CDC)'s Division of Vital Statistics released a recent report titled *Births: Provisional Data for 2017*, which placed the provisional general fertility rate (GFR) at approximately 60.2 births per 1000 women aged 14–44. This rate is reported to be down 3% from 2016 and a record low for the United States. The provisional total fertility rate (TFR) which is based on the age-specific births in a given year, and estimates the number of births that a hypothetical group of 1000 women would have over their lifetimes, is also down 3% from the rate reported in 2016— the lowest TFR since 1978 [1]. Though fertility rates are a multifaceted

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phenomenon, recent findings regarding reproduction and family planning may be contributing to the increasingly low rates. For instance, a recent populations report by the U.S. Census Bureau found that young adults, aged 18–34, not only believe that economic and educational accomplishments are far more important milestones than marriage and parenthood, but that they are also actively delaying parenthood [2]. Moreover, a study on the temporal trends in sperm count also found that the average sperm counts for men, unselected for their fertility status, has declined in western countries by approximately 59% since 1973 [3]. This is the most comprehensive study to date that not only shows the continuous nature of the decline but also calls for the urgency in male reproductive research, as such findings have significant public health implications. Combine this with the increasing number of cancer survivors (both post and pre-pubertal patients) that also require fertility preservation therapies [4]. Currently, the only option available for male cancer patients is based on successful sperm retrieval and sperm freezing for future use. This is an option not extended to prepubertal patients, as these individuals have not yet produced viable sperm, and are left with no other alternatives to conceive children for their entire life. Altogether, the overall cultural shifts in family planning alongside the declining rates of the general fertility has led to major market research reports expecting the global fertility services market to grow from the current multimillion dollar industry to upwards of $30 billion. Therefore, the advancements in fertility services are increasingly significant in our changing global populations.

The production of germ cells (i.e. gametogenesis), is a process that begins in embryos with the formation of primordial germ cells (PGCs) that continues differently in male and female reproductive systems. Only recently did studies show that mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can differentiate into PGC-like cells (PGCLCs) that upon transplantation gave rise to both functional sperm or oocytes [5, 6]. Importantly, the culture conditions and differentiation protocols of murine PGCLCs still require further optimization, as these cells differentiate inefficiently and lack well-defined long-term culture conditions [7]. This is especially the case with SSCs that constitute the male testis. Understanding how these stem cells initiate spermatogenesis within the seminiferous tubules of the testis is vital for the future of IVG applications, as such knowledge would lead to optimized differentiation protocols and long-term SSC culture conditions that could be implemented for the treatment of infertility [8].

The focus of this chapter is on the significance of mammalian spermatogenesis as it pertains to both the successes of IVG, and to the push for innovation in the general field of reproductive medicine. We will see how SSC fate decisions establish spermatogenesis through the multifaceted interaction of the stem cell and its niche. Furthermore, we discuss how novel technologies, which allow for SSC niche mapping and in vitro preservation of the seminiferous tubules, hold the key to therapeutic and diagnostic breakthroughs—and the challenges and ethical implications that follow.

2. The spermatogonial stem cell niche establish and regulate spermatogenesis

The variation in stem cell behavior, whether it is the decision to self-renew or to commit to differentiation, is strictly linked to the stem cell's niche [9]. The niche is highly dynamic and has

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shown to direct such fate decisions in a variety of adult stem cells such as hematopoietic stem cells (HSCs) [10], intestinal [11], and epidermal stem cells [12]. The concept of the niche extends beyond the direct cell–cell contact and is comprised of other key components such as secreted factors (i.e. chemokines, hormones, growth factor receptors), inflammation (i.e. macrophages, T cells), physical factors (i.e. shear forces, topography, elasticity/stiffness), hypoxia (i.e. glycolysis-optimizing conditions), and cellular metabolism (i.e. glucose, lipids, calcium, calcium receptors) [9]. Importantly, the stem cell-niche communication not only occurs over short and long range distances, but is also reciprocal and significant in tissue homeostasis [13–15]. For instance, in mouse skin, the removal of the stem cell population (i.e. hair follicle stem cells) can result in niche cells dedifferentiating to replace them. In this case, the repopulation of epithelial cells (cells that do not contribute to hair growth), replace the stem cells and sustain hair regeneration [16]. Interestingly, with respect to the field of spermatogenesis, the key components of the SSC niche also appear to be as multifaceted as the examples provided above.

2.1. Direct and indirect cell contact: SSCs, Sertoli cells, and other key players

One key player in regulating the accessibility of SSCs to other components in the niche is the Sertoli cell. These cells extend from the basal compartment of the seminiferous tubule to the adluminal region. Sertoli cells take on a constantly evolving and irregular shape that is in a continuous three-dimensional relationship with not only the SSCs, but also the differentiating spermatogonia throughout spermatogenesis [17]. Due to the complexity of such a dynamic relationship, traditional experiments involving two-dimensional or stage-specific cell analysis may have portrayed an incomplete depiction of spermatogenesis and may have attributed to the large discrepancy found in today's literature [8]. Regardless, it is still worth discussing such findings because it provides a glimpse into the three-dimensional relationship between the SSC and Sertoli cells.

To begin, Sertoli cells have a large surface area that allows them to support germ cell development at a higher ratio of germ cells to Sertoli cells [17, 18]. Such a characteristic is critical for providing structural support to the germ cells, but also to germ cell movements throughout the tubule. Furthermore, the unique structural and signaling flexibility of Sertoli cells create two distinct environments within the tubules that are otherwise referred to as the blood-testis barrier (BTB). This is where the basal compartment, the region in close contact with lymph and blood, is speculated to maintain earlier staged cells of spermatogenesis, such as SSCs and early progenitors. Later stages that are committed and differentiating spermatogonia, however, appear to occur in the adluminal compartment, isolated from lymph and blood [17]. Importantly, the BTB is created by tight or gap junctions, and desmosomes that are present between Sertoli cells. The BTB does not exist between Sertoli cells and germs cells or between germ cells [19–22]. Additionally, the specialization of the BTB was first depicted in early studies of testicular transferrin. Cells located in the later staged adluminal region of the tubule (i.e. cells that do not have access to serum iron), gained access to testicular transferrin through Sertoli cells [17, 23, 24].

In addition to the role of the Sertoli cells, Leydig cells within the interstitial space of the seminiferous tubules produce and signal testosterone. Without the presence of testosterone, spermatogenesis does not proceed completely and results in male infertility. Therefore, if Leydig cells were to be removed, the germ cells that have initiated meiosis and completed

differentiation begin to improperly detach from the Sertoli cells and die [25]. Furthermore, mature sperm near the lumen of the tubule (i.e. the adluminal compartment) cannot properly release from Sertoli cells without testosterone signaling. Another major hormone, follicle-stimulating hormone (FSH) has shown to act synergistically with testosterone to increase and regulate spermatogenesis. It does this by binding to the FSH receptor (FSHR) on the surface membrane of Sertoli cells [25]. Peritubular myoid cells (PMCs) are also located in the interstitial space of the tubules, and express the androgen receptor for testosterone. Within the seminiferous tubules, however, only Sertoli cells express the androgen receptor. Therefore, Sertoli cells, in communication with other niche cells (i.e. Leydig cells and PMCs) regulate spermatogenesis indirectly and thus impact the initiation, development, and survival of germs cells [25, 26]. Furthermore, the surface of some seminiferous tubules associates with vascular endothelium and perivascular cells [27]. The role of such vasculature-associated cells may be involved in regulation of SSC niche dynamics. For instance, some studies found that in the prepubescent and adult testis, macrophages are closely associated with Leydig cells and play a role in the signaling and production of testosterone [28]. Another study found populations of macrophages near the surface of the basal compartment where enriched undifferentiated spermatogonia were found. Such testicular macrophages expressed SSC proliferative and differentiating factors such as enzymes involved in retinoic acid (RA) synthesis and colony stimulating factor 1 (CSF1) signaling [27].

2.2. The extracellular matrix, secreted factors, and their respective receptors

The extracellular matrix (ECM) and its role in the stem cell niche vary substantially in almost every tissue [9]. In some cases, the ECM is also involved in the maintenance of local concentrations of growth factors that direct stem cell fate or target niche cells involved in the regulation of those SC fate decisions [29, 30]. In the testis, the ECM located at the basal compartment, is made up of the basement membrane that is composed of proteins like laminin, type IV collagen, and entactin. Importantly, the basement membrane (a modified ECM) is speculated to not only interact with Sertoli cells, but also regulate SSC fate decisions. Sertoli cells even secrete components of the ECM (i.e. laminin) that is not only useful in short-term SSC culture conditions, but is also involved in Sertoli cell tight junctions, and in turn, the formation of the BTB [8, 17, 31–33]. It is the formation of tight junctions across Sertoli cells that is said to create a semipermeable barrier that restricts molecule movement based on either weight or chemical structure [34]. The exact involvement, however, of the basement membrane with key components like laminin and the mechanisms of junction formation still require further investigation with their role in SSC niche dynamics. Nevertheless, specialized junctions have been found throughout the seminiferous epithelium and include junctions such as adherens, desmosome-like, hemidesmosome, and gap junctions that are located throughout the tubule from the basal to adluminal compartment. These junctions not only appear to control germ cell movement, but are also involved in the regulation or perhaps local concentrations of secreted factors in SSC fate decisions [17].

Glial cell line-derived neurotrophic factor (GDNF) is a secreted factor produced by Sertoli cells and PMCs that is linked to SSC fate determination, Sertoli cell proliferation and short-term

SSC *in vitro* maintenance [35–37]. Furthermore, the *in vitro* GDNF expression promotes the self-renewal and proliferation of SSCs by activating the phosphoinositide-3 kinase (PI3K)/AKT signaling pathway [38]. Studies *in vivo,* however, show that ERK1/2 signaling pathway is activated in SSC self-renewal, while PI3K/AKT signaling is shown to be activated in SSC proliferation during stages where RA signaling is both low and high [39]. Based on the significance of Sertoli cells as a key player in the niche, one can suspect that such cells and their respective GDNF expression is a relationship that is sufficient for the recapitulation of niche dynamics for *in vitro* studies. The current discrepancy in the literature, however, suggests that once SSCs are removed from *in vivo* conditions, the multifaceted three-dimensional SSC niche is then disrupted in a significant way that causes SSCs to behave differently, and perhaps inconsistently. The establishment of a long-term and well-defined *in vitro* protocol for SSC maintenance would no longer be an open question if the maintenance of normal spermatogenesis were based on a fairly simplified stem cell niche model. In fact, recent reports appear to provide further support for a niche that is in a three-dimensional relationship with the SSCs. For example, a recently published report found that cyclic expression of GDNF is not only required for SSC homeostasis but also that GDNF cyclic expression is normally expressed during spermatogenesis [35]. Furthermore, the ectopic expression of GDNF during late staged spermatogenesis caused the accumulation of early-staged undifferentiated spermatogonia that was also positive for the GDNF receptor (GFRA1). Another study found that the lack of a RA target gene in mice called Stimulated by Retinoic Acid gene 8 (Stra8), caused the accumulation of undifferentiated spermatogonia and the depletion of differentiating spermatogonia. Furthermore, the capability of germ cells to begin differentiation or meiotic initiation in response to RA was distinct, periodic and limited to a particular seminiferous stage. This study suggests that properly timed differentiation depends on the intersection of cell intrinsic competence and extrinsic chemical cues. Such findings led to the conclusion that periodic RA-STRA8 signaling intersects with the periodic germ cell capability to regulate spermatogenesis [40].

In terms of respective receptors, in the seminiferous epithelium, some major ECM-receptors are integrins. In fact, studies have claimed that integrin-α6 and integrin-β1 are key surface markers involved in the regulation of spermatogenesis. There is still much discrepancy, however, on whether such markers are exclusively expressed on SSCs or germ cell progenitors [8, 17]. It has been shown, however, that the deletion of integrin-β1 on Sertoli cells not only reduced SSC homing (i.e. the repopulation of SCs after the removal of endogenous SCs), but also that the adhesion receptor’s association with laminin is critical for the several steps involved in SSC homing [41]. Again, though the integrins are significant in niche dynamics, it appears that integrins cannot be used to distinguish the sub-populations of early staged germ cells (i.e. SSCs and their progenitors). Importantly, such studies use marker-based techniques (i.e. Fluorescence-activated cell sorting (FACS)) to isolate cells positive for markers such as integrin-α6, integrin-β1 or GFRα1, to then transplant back into germ cell-depleted testes for further analysis [8, 42, 43]. Though the transplantation assay [44] is a great tool to gauge stem cell competency, the SSC niche dynamics must also be clearly defined since germ cells have shown to behave inconsistently from *in vivo* to *in vitro* conditions. Therefore, perhaps the discrepancy in such studies highlights the limitations or inability of marker-based techniques,

like FACS, to suffice for the understanding of the SSC-niche relationship. Such a variation in SSC behavior is further highlighted in a study that modeled the entire GFRα1+ population within the seminiferous tubules. Results showed that during steady-state spermatogenesis, the GFRα1+ population comprised of a single stem cell pool that continually interconverted between different states of equipotent singly or syncytial states [45]. The early example of the hair follicle stem cells further resonates with this study because self-renewal potential may be influenced by the position of the stem cell within the niche. Another recent publication also attempted to address the heterogenous expression of markers present on early staged germ cells comprise of stem cells and progenitors. More specifically, the isolation of GFRα1+ and GFRα1-spermatogonia, interestingly, both showed elevated transplantation activity. Furthermore, GFRα1-spermatogonia not only produced GFRα1+ spermatogonia when negative cells were transplanted into germ cell depleted testis, but also restored spermatogenesis. Such results indicated that a stem cell pool of GFRα1+ and GFRα1-cells could interconvert between the two states of positive and negative cells in a niche-dependent mechanism. Additionally, though these two populations may be closely related, they still differ in key cell-intrinsic components [46]. Altogether, both studies display the behavioral variation in not only GFRα1+ germ cells, but also GFRα1+/− populations in relation to the niche. The GFRα1+ only population interconverts between different stem cell states while the GFRα1+/− populations reveal a niche-dependent mechanism for fate determination. How these two studies come together for a more cohesive story requires further investigation and an overall better niche understanding.

2.3. Hypoxia, metabolism and the role of inflammation

Tissue specific cell populations such as HSCs, and cardiac progenitors are found to be in low oxygen (i.e. hypoxic) microenvironments that contribute to cell survivability and maintenance [9, 47]. During hypoxic conditions, cells favor glycolysis rather than mitochondrial oxidative phosphorylation. In terms of the SSC niche, one recent report found that the reduction in O_2 tension during *in vitro* conditions enhanced the *in vivo* maintenance of the SSCs' regenerative integrity. SSCs cultured long term in hypoxic conditions (10% O_2 tension rather than the standard 21% O_2 tension), had the capacity to continue spermatogenesis following transplantation in recipient tubules devoid of germ cells [48]. Previous work from the same group had also shown that key glycolysis regulating enzymes were elevated in cultured undifferentiated germ cells [49]. Furthermore, another study found that the inhibition of glycolysis (via the double-knockout of Myc/Mycn genes) decreased SSC activity and inhibited spermatogonial differentiation. The chemical stimulation of glycolysis, however, increased the frequency of the SSC self-renewal capacity [50]. On the contrary, another report found that the ablation of Max (a key partner for Myc function) induced differentiation of germ cells in culture conditions [51]. Interestingly, the same group that reported on SSC self-renewal through the chemical activation of glycolysis also reported on a lipid-rich medium that enhanced SSC self-renewal for long-term culture conditions [52, 53]. The excess of free fatty acids, however, promotes fatty acid catabolism (i.e. β-oxidation and oxidative phosphorylation) rather than glycolytic activity [48]. Together, there are possible explanations and takeaways from such discrepancies regarding hypoxia and metabolism. First, low oxygen-tension may be

involved in the regulation of both *in vitro* and *in vivo* SSC self-renewal. Furthermore, though examples of HSC-niche dynamics have shown that hypoxic microenvironments contribute to stem cell maintenance, this in some ways contradicts the role of the BTB in spermatogenesis. The regions where early staged spermatogenesis occurs are in the basal compartment of the tubule, near lymph and blood. One would then suspect that the basal environment favors oxidative phosphorylation over glycolysis. Another possibility, however, is that the Sertoli cell tight junctions may create a highly restrictive, thus semi-hypoxic, niche for the SSCs in order to tightly regulate self-renewal. Second, there is also the possibility that the connection of low oxygen-tension to SSC maintenance may only be an *in vitro* phenomenon. As previously stated, the three-dimensional relationship of the niche to the SSCs may be significant enough to cause these cells to behave differently once removed from *in vivo* conditions. For instance, the disruption of this multifaceted communication may cause SSCs to begin to favor glycolytic activity during *in vitro* conditions. Furthermore, reactive oxygen species (ROS) were shown to also influence the outcome of *in vitro* SSC maintenance. Moreover, modulating ROS levels demonstrated that moderate concentrations may promote SSC self-renewal and that achieving this may be possible by manipulating or reducing the use of β-oxidation as the primary bioenergetics pathway [48, 54]. Such speculations, however, only point to the need for further investigations of the three-dimensional relationship of the SSC and its niche. Overall, cellular metabolism is critical in determining whether stem cells proliferate, differentiate or remain quiescent. There is typically a balance that is established between oxidative phosphorylation, glycolysis, and oxidative stress for the adult stem cells [9]. Such a balance is further exemplified in the dynamic niche with the role of inflammation.

The general understanding regarding immune privilege (i.e. the capacity to tolerate the introduction of new antigens without the trigger of an inflammatory response) is the evolutionary adaptation to protect tissues from loss of functions due to their limited capacity for regeneration [55, 56]. In the testis, however, this protection against loss of function is for the tissue's reproductive capacity. The production and differentiation of male germ cells are unique in that sperm matures at puberty, which is long after the maturation of the immune system and systemic self-tolerance [55]. Due to these phenomena, the BTB plays a role in protecting the maturation of sperm from an autoimmune reaction. For instance, the various junctions and desmosomes between Sertoli cells have created such a limited access in the passage of other molecules that the composition of the basal, adluminal, and interstitial spaces differ significantly. Additionally, while the BTB's ability to isolate meiotic and postmeiotic germ cells from lymph and blood is significant in testicular immune privilege, there are also physical and immunological components required for the immunotolerance of the testis [55]. The expression of anti-inflammatory cytokines by immune and somatic cells and the role of androgens also play a role in the immunoprivileged niche.

Meiotic and postmeiotic germ cells express a large variety of neoantigens that emerge during puberty and long after self-tolerance is already established. Furthermore, once spermatogenesis begins, the BTB is established and immediately isolates post pubertal germ cells from the immune system [55]. Interestingly, germ cell neoantigens are also present on SSCs and progenitors that are located in the basal compartment, and not isolated from lymph and blood, unlike the adluminal compartment of the BTB [55, 57, 58]. This suggests that other

components of the testes also play a role in the immunoprivileged niche. For instance, accumulating evidence suggests that PMCs secrete cytokines like transforming growth factor-β (TGF-β), leukemia inhibitory factor (LIF), and macrophage chemoattractant protein 1 (MCP-1) that directly affect leukocytes in the interstitial space of the testis [59, 60]. Furthermore, the local high concentrations of testosterone appear to play an important role in the immunoprivileged niche within the testis. For example, when testosterone was incubated with stimulated human macrophages, monocytes and non-immune cells, the suppression of cytokines and adhesion molecules occurred while the production of anti-inflammatory cytokines increased [61, 62]. In transplantation studies, rats that were treated with estrogen to suppress Leydig cell production of testosterone immediately rejected allotransplanted cells within the seminiferous tubules. This directly contrasted the untreated control group where no rejection occurred in allotransplanted cells [55]. Though this provides evidence that the local high concentrations of testosterone plays an important role in the immunoprivileged niche, the exact mechanism testosterone and its anti-inflammatory function on testicular leukocytes, still remains unknown. There may be an indirect regulatory mechanism involved in the balance between the expression of pro and anti-inflammatory cytokines in Sertoli cells, Leydig cells, and PMCs [55]. Such a balance between pro and anti-inflammatory cytokines may also play a significant role in the protection and maintenance of SSCs that are not isolated from lymph and blood. For instance, *in vitro* studies have shown that rat testicular macrophages exhibit immunosuppressive characteristics [63]. Interestingly, a heterogeneous macrophage population resides in the rat testis' interstitium where one population participates in the inflammatory response while the other is thought to have a role in the immunoprivileged niche [63, 64]. This suggests that the testis is capable of initiating both a normal inflammatory response and maintaining an immunoprivileged niche. Such a balance appears to be crucial in the SSC niche, especially since there is mounting evidence of immune-related male infertility [65, 66]. Importantly, the microvasculature in the interstitium not only contains macrophages, lymphocytes, and mast cells but also dendritic cells (DC). Studies have shown, thus far, that testicular DCs may play a significant role in maintaining the balance of testis tolerance and intolerance [63, 67], but still require further investigation. The role of inflammation and the extent of its connection to SSC function are still unclear. One would speculate, however, that because the immune privileged testis protects the tissue's reproductive capacity, that this may also directly connect to SSC fate decisions.

2.4. Physical factors

The physical surroundings such as the three-dimensional physical shape, shear forces, and topography (i.e. the physical arrangement of cells) all contribute to the stem cell and its niche [9]. For example, shear forces such blood flow, have shown to play a role in either the acceleration or reduction of *in vivo* development of zebrafish embryonic HSCs [68]. Distinct niche topographies have also shown to have an effect on signaling pathways and the regulation of differentiation on mesenchymal stem cells [69]. Testicular blood flow, and its connection to niche dynamics and spermatogenesis, is largely an under-investigated area. Interestingly, older reports showed that a reduction of approximately 70%, for 5 hours, from normal blood flow led to varying degrees of damage to the seminiferous tubules and

resulted in an inflammatory-like response (i.e. increased number of leukocytes within the testis interstitial space was found) [70]. Additionally, one important suggestion from this study was that vasomotion, the smooth muscle oscillations of the blood vessel walls (independent of heartbeat), contributed to testicular function. Some studies even found that vasomotion was directly regulated by testosterone, and indirectly by Sertoli cells [71, 72]. More specifically, vasomotion was not detected in the testes where Leydig cells were removed but was induced with a dose of testosterone [72]. More recently, however, one report found that Sertoli cells may play a direct role in supporting the testicular vascular network. Results showed that loss of germ cells had no effect on testicular vasculature while loss of Sertoli cells led to the reduction of total vascular branches, volume, and the number of small micro-vessels [73]. The mechanism by which Sertoli cells influence vasomotion still remains unclear. Perhaps the multifaceted communications between Sertoli cells, Leydig cells and PMCs also work to regulate vasomotion.

Furthermore, the biophysical cues involved in the *in vivo* ECM, including the basement membrane and the formation of the BTB, are all significant factors in the SSC niche topography. Equally importantly, however, is the distribution of germ cells within the seminiferous tubules. One study found that the distribution of early mouse germ cells, typically termed type A spermatogonia that are either a single cell, paired or aligned, within the spermatogenic cycle is nonrandom [74]. Interestingly, the transplantation of a single SSC first results in asymmetrical spermatogenesis followed thereafter by uniform spermatogenesis (i.e. spermatogenesis around the entire tubule) [74, 75]. What this suggests is that SSCs are capable of movement. Further evidence provided by Chiarini-Garcia *et al.* indicated that spermatogonia were not only mobile, but also cyclically positioned themselves at periodic intervals along the tubules to ensure uniform spermatogenesis. Such a nonrandom distribution of germ cells was made possible because tubules remained in somewhat of a constant relationship with each other. This contact with one another also allowed the same group to map the topography of these tubules [74]. Together, one can speculate that the three-dimensional physical shape and the biophysical cues of the testis may be significant in the SSC niche dynamics. The physical factors involved in the SSC niche, however, appear to be undervalued in the field of spermatogenesis as many questions remain to be unanswered.

2.5. Niche mapping and its significance in the seminiferous tubule microenvironment

Modulating the SSC niche requires well-defined and reproducible studies. Since SSCs appear to be highly interconnected with their niche, the *in vivo* dynamics must be clearly defined, and more importantly, encompass all the key components of the stem cell-niche interactions. Just as Handel *et al.* made the necessary case for applying 'gold standards' (i.e. benchmarks) to *in vitro*-derived germ cells, we make the case for extending and expanding such benchmarks in the studies involving spermatogenesis. Handel *et al.* made the vitally important argument for the scientific community to apply the highest standards in evaluating and conducting IVG research. To further emphasize their point, we believe that much of the discrepancy in the literature today is due the lack of consensus or a laid out rigorous criteria by which

to evaluate SSC identity, SSC fate decisions, and the general understanding of mammalian spermatogenesis, as it is the foundational knowledge for future IVG practices. Moreover, Lane *et al.* effectively organized the composition of the general stem cell niche into reciprocal interactions of different cellular components, secreted factors, ECM, immunological control, metabolic control and physical factors that we sought out to re-evaluate and highlight some of the literature in the field of spermatogenesis, under that same style of organization. As the literature shows, SSC niche dynamics appear to include all of those reciprocal interactions. Our understanding of spermatogenesis, however, still remains incomplete due to perhaps the lack of aerial or three-dimensional perspective with regards to not only the types of experiments conducted, but also the interpretation of those results. SSCs, and niche cells alike, appear to behave much differently during *in vitro* conditions than *in vivo* dynamics as much of the inconsistencies in the literature suggests. Aside from the variability in culture protocols that may contribute to this dilemma, we believe two actions are required for the push forward in the field of spermatogenesis. First, it is imperative for a set of benchmarks to be put into place when evaluating the *in vitro* recapitulation of spermatogenesis. Perhaps this involves the modification of Lane *et al.'s* organization of the stem cell niche to the SSC niche dynamics as summarized pictorially in **Figure 1**. Second, emerging methodologies and technologies may be required to facilitate the implementation of better *in vitro* analyses of spermatogenesis.

A recently published report characterized a three-dimensional multilayer model (termed the Three-Layer Gradient System (3-LGS)) that allowed for the reorganization of dissociated rat testicular cells into testicular organoids with the formation of a functional BTB and germ cell maintenance. This system used three concentric layers of Matrigel to not only increase

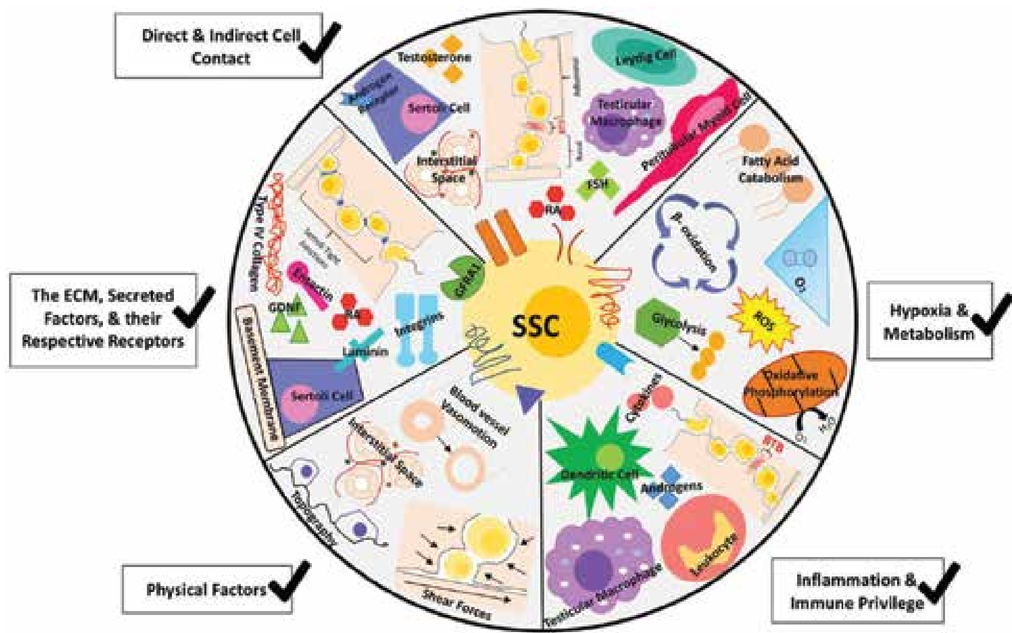

Figure 1. The spermatogonial stem cell and its niche, reimaged.

the area for factor exchange but also for testicular cell reorganization into organoids to take place [76]. Furthermore, the cellular organization of this *in vitro* three-dimensional multilayer model more closely represented the *in vivo* stem cell-niche interactions. This group also proposed that the 3-LGS is a new platform to better investigate the SSC niche *in vitro* [76, 77]. Interestingly, such an *in vitro* three-dimensional multilayer model represents an emerging methodology that not only signified the three-dimensional relationship of the SSC-niche interactions, but also provided a potential avenue to further study the SSC niche in a working *in vitro* model. Alongside the more appropriate development of three-dimensional *in vitro* models, methodologies that can image three-dimensional *in vivo* conditions still remain either undervalued in the field or lack the technological capabilities for in-depth analysis. For instance, though some work has been done using time-lapse imaging and histological staining of the seminiferous tubules, high quality data and clear analysis of the undisturbed *in vivo* environment is still a clear hurdle [78]. This same challenge remains in general biology, where obtaining high-resolution information from a complex system without losing the global perspective required to understand how the system functions, is still lacking [79]. Tissue clearing technologies, however, a technique developed for brain mapping [80] may address such challenges, especially in spermatogenesis where three-dimensionality appears to be highly significant. Through chemical transformations, this method (i.e. CLARITY) passively or actively transitions the whole tissue into a lipid-extracted, thus clear, and structurally stable tissue that is fully intact with its native biological molecules [81]. Brain mapping has significantly advanced due to technological innovations in tissue clearing methodologies like CLARITY [81]. The uniqueness of such a technique not only enables clear accessibility, but also maintains a global perspective in a three-dimensional analysis. Perhaps the mapping of complex systems through tissue-clearing technologies can also be expanded to the SSC niche dynamics (i.e. SSC niche mapping).

3. Recapitulating *in vivo* spermatogenesis through *in vitro* preservation

The *in vitro* propagation and maturation of germ cells is especially necessary for the development of therapies for prepubertal pediatric patients undergoing chemotherapy. Typically the storage of sperm for cancer patients has become routine since therapeutic agents, like radiation and chemotherapy, either directly or indirectly affect the SSC pool and ultimately patient fertility. Such options, however, do not extend to pediatric patients, as those individuals have not yet produced viable sperm [4]. Therefore, an alternative approach to studying spermatogenesis has shown great promise through the use of organoid static conditions or three-dimensional fluidic conditions. For instance, the *in vitro* production of functional sperm from neonatal mouse testes, using a well-defined organ culture protocol, was significant in providing a new avenue for *in vitro* spermatogenesis [82]. This study not only maintained organoids for approximately 2 months to obtain spermatids and sperm, but also produced healthy and competent offspring through micro-insemination. The system, however, still lacks the controlled monitoring necessary for the manipulation and study of SSC niche dynamics [83]. Furthermore, the use of testis fragments (i.e. testicular organoids) in static

conditions has limitations on the tubule maturation and tubule viability since the diffusion of nutrients and oxygen are constrained by the lack of a functional vascular system [84]. In order to address such limitations, the same group developed a different approach to *in vitro* spermatogenesis. A simple microfluidic device with a porous membrane was created to mimic the microvascular system of the testis [85]. The porous membrane separated the tissue from the flowing medium and successfully maintained spermatogenesis for approximately 6 months. Furthermore, the seminiferous tubules of testis were spread flat in the microfluidic chamber and in direct contact with the porous membrane. Interestingly, germ cell count, and thus, differentiation decreased over time in the long-term microfluidic culture. This may be the result of poor homeostasis between the tissue and the medium [83]. Tissue homeostasis is key in modulating the stem cell niche and the homeostatic imbalance may have also resulted from the distortion of the tubule-to-tubule contact found *in vivo.* Furthermore, perhaps the tubules spread flat in the chamber led to the loss of the interstitial fluid and niche cells, such as Leydig cells and PMCs that could have also contributed to the decrease in germ cells over time.

Together, the controlled monitoring of a fluidic *in vitro* system that uses testicular organoids is significant. Preserving the complete SSC niche in a fluidic platform, however, is something that has not yet been successfully achieved. The static organoid culture is limited to oxygen and nutrient diffusions and cannot maintain overall tubule viability (i.e. organoid necrosis typically found in the center of testis fragments during static culture). Furthermore, the recent publications on microfluidic culture protocols also distort the three-dimensional environment of the testis that ultimately affects SSC maintenance [85, 86]. An optimized fluidic platform that will not only recapitulate the SSC niche, but also allow for the easy manipulations of SSC niche dynamics may be the next step for *in vitro* spermatogenesis.

4. Initiating innovation: promises, challenges, and the ethical implications for the push toward IVG

Human embryogenesis and gametogenesis is crucial to our understanding of reproduction, development, disease and evolution [87, 88]. The recent successes in the generation of human PGCLCs from human ESCs and human iPSCs solidified the prospects that the reconstitution of human IVG may be near [7]. In the future, IVG combined with IVF can allow infertile couples trying to conceive to generate their own gametes through iPSC technology [7, 89]. Interestingly, IVG has the potential for even broader implications in reproductive medicine. With the increasing cultural shift in family planning, such as the delay in marriage and parenthood from young adults, is a shift that will most likely impact the GFR and the replacement fertility rate in any given country. Countries that have a low GFR have a larger aging population that is not only positioned to shrink, but also has fewer populations in the working age to support the older dependents. Therefore, the replacement fertility rate, highly dependent on the GFR, is the rate in which women give birth to enough babies to sustain population levels in any given country [90]. With the availability of contraceptives, young adults can now easily delay parenthood to ages where fertility begins to decline and conceiving may become more difficult and at times no longer possible. IVG/IVF therapy combined with iPSC technology

can potentially provide a solution to the significant implications in the new cultural shifts that will affect the GFR and the replacement fertility rate across countries. Until such a promising therapy can come into fruition, there are many scientific and ethical challenges that we must undertake. The need for robust and reproducible studies in both spermatogenesis and oogenesis are crucial for the future in IVG practices. It is imperative to evaluate claims of IVG and germ cell meiosis through the use of standardized benchmarks and the demonstration of the 'gold standards' for meiosis [91]. Furthermore, the advancement in both knowledge (i.e. understanding SSC niche dynamics) and technology (i.e. creating comprehensive fluidic devices and viable *in vitro* platforms) are still clear challenges that need to be addressed.

In terms of the ethical implications of IVG, it is imperative for us to revisit the relevant regulatory measures. For instance, 10 countries permit human embryo research under the 14 day culture rule [87]. Of these countries, not including the United States, only seven include regulations to human IVG for medical or scientific applications. Interestingly, though the United States has no federal laws or regulations to prohibit IVG for research, the Dickey-Wicker amendment (signed in 1995) forbids federal funding for human embryo research [87, 92]. Even though the National Institutes of Health (NIH) recognized the value of it, and the CDC continues to report the GFR at record lows. Combine this with the 2017 provisional CDC report that birth rates are declining for nearly all age groups of women under 40, but rising in women aged 40–44 by 2% from 2016 [1]. Furthermore, just as the development of IVF was initially highly controversial, it has now become a widely accepted treatment for infertility, and more commonly used among women with declining fertility. Therefore, if the scientific and societal value of human IVG research is agreed to be significant, IVG research should then be conducted under balanced regulations with careful ethics review and close oversight [87]. Scientists, appropriate policymakers and the public should all be included for all future discussions regarding human IVG research.

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Chapter 3

The Adipokines in Domestic Animal Reproduction: Expression and Role in the Regulation of Ovarian Function

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Additional information is available at the end of the chapter

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Abstract

Currently, it is clear that female reproduction is regulated by the hypothalamic–pituitary-ovary axis, which produces many hormones that control reproductive stages. It is therefore important to have knowledge of new regulators/hormones controlling reproduction in domestic animals. In female animals, energy metabolism and fertility are tightly connected, and reciprocally regulated. The adipose tissue is well known to be implicated in the secretion of several hormones, such as the adiponectin, resistin, chemerin, visfatin, vaspin and apelin, the so-called adipokines or "adipose tissue-derived hormones". Many reports indicate that adipokines regulate the ovarian follicles' development, the onset of puberty and/or ovulation. This chapter summarizes that several adipokines are expressed in the ovary and that they can regulate ovarian physiology such as the steroid hormone production, cell proliferation, apoptosis and oocyte maturation in different domestic animals like pigs, cows, goats, ewes, chickens and turkeys.

Keywords: ovary, expression, steroidogenesis, proliferation, adipokines, domestic animals

1. Introduction

The reproductive system in female domestic animals is regulated precisely by an intricate interplay of hormones produced by the hypothalamus, anterior pituitary and the ovaries. The interplay of hormones results in ovarian cyclicity in females, which in consequence leads to fertilization, delivery by the maintenance of pregnancy of offspring. Moreover, it is now clear

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that fertility depends on the energy metabolism status. For example, in cattle, genetic selection for high milk production is associated to high negative energy balance in the post-partum period and reduced fertility [1]. In pigs, a negative energy balance and a decrease in body fat results in a reduction in litter size and viability of piglets [2]. In sheep, it is well known that an increase in availability of energy substrates is associated with an increase in prolificacy [3]. Furthermore, obesity and some metabolic disorders influence the reproductive hormones in women [4].

The adipose tissue is well known to be implicated in the secretion of several hormones such as adiponectin, apelin, chemerin, resistin, vaspin and visfatin: the so-called adipokines, which regulate energy balance, food intake, immunology and diabetes. A recent study indicated that several adipokines are expressed in the ovarian cells and that they can modulate ovarian physiology in some domestic animals, like pigs, cows, goats, ewes, chickens and turkeys [5].

2. Short description of adipokines

Adiponectin, apelin, chemerin, resistin, vaspin and visfatin were described as “adipose tissue-derived hormones”. However, their expression and receptors are present in different tissues like the brain, stomach, kidneys, pancreas, liver or blood vessels. They play important roles in several metabolic processes, such as in the regulation of insulin sensitivity, food intake, adipogenesis and inflammation (**Figure 1**).

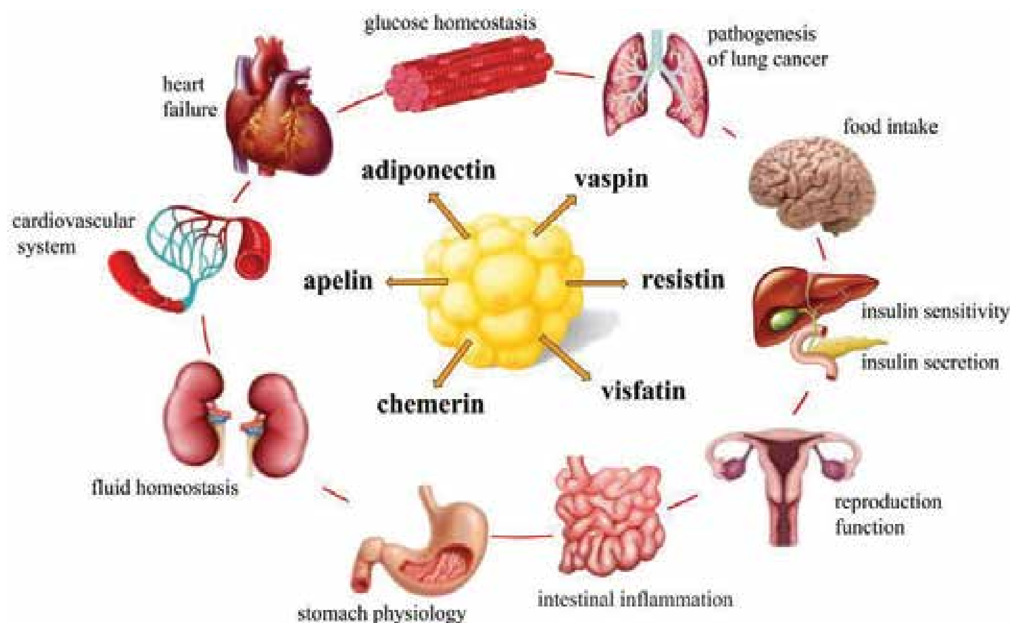

Figure 1. Adipokines and their receptors are present in different tissues like the brain, stomach, kidneys, pancreas, liver or blood vessels, and play important roles in several metabolic processes.

2.1. Adiponectin

Adiponectin is mainly produced by a white adipose tissue (WAT) and secreted into the bloodstream. The adiponectin level in serum is inversely related to body weight. Circulating levels range between 2 and 30 μg/ml in human plasma and are higher in females than in males [6]. In chicken plasma, the adiponectin levels are in the range of 4–10 μg/ml. The protein (26 kDa) was described for the first time by Scherer et al. [7] and is present in cells and plasma in three forms: trimers, hexamers and high-molecular weight (HMW) [8]. A number of post-translational modifications are required to obtain these forms. Three adiponectin receptors have been identified: AdipoR1, AdipoR2 and T-cadherin. The first two are the main adiponectin receptors and consist of seven transmembrane domains. AdipoR1 is abundantly expressed in skeletal muscles and is associated with the activation of AMP-activated kinase pathways. AdipoR2 is mainly expressed in the liver and is associated with the activation of peroxisome proliferator-activated receptor (PPAR)-α pathways. Then, T-cadherin is a receptor for hexameric and HMW adiponectin and is expressed on vascular cells and smooth muscles [9]. Adiponectin is an insulin-sensitizing, vascular protective, anti-apoptotic, anti-lipotoxic and anti-inflammatory protein on different cell types. Thus, it has been considered as a beneficial adipokine. Moreover, these functions mark this protein as a potent drug targeting diabetes and obesity-associated diseases [10].

2.2. Apelin

Apelin has been isolated from the bovine stomach extracts as an endogenous ligand of the previous orphan receptor APJ [11]. APLN gene encoding human apelin is located on chromosome Xq 25–26 [11]. Preproapelin, a precursor of the mature form, consists of 77 amino acids and is transformed into active forms by enzymatic hydrolysis. They are distinguished within four forms having different biological activities: apelin-36 (preproapelin 42–7), apelin-17 (preproapelin 61–77), apelin-13 (preproapelin 65–77) and pyroglutamate-apelin-13 (Pyr-apelin 13) [11]. The shorter apelin-13 has much higher biological potency than the longer apelin-36. Mature apelin is apparently the only monomeric protein without cysteine residues which occur in the precursor [12]. This adipokine was described in various tissues and organs such as the uterus, ovary, heart, lung, stomach and brain. The expression of apelin increases during adipocyte differentiation. Insulin, growth hormone (GH) or tumor necrosis factor (TNF-α) are among the agents regulating the production of apelin [13]. Apelin exerts some influence on the cardiovascular system, food intake, fluid homeostasis and energy metabolism. According to Castan-Laurell et al. [14], lean women exhibit plasma apelin levels in the range of 272 pg/ml, indicating a positive correlation between the plasma apelin level and body mass index (BMI).

2.3. Chemerin

Chemerin was first termed tazarotene-induced gene 2 protein (TIG2) and retinoic acid receptor responder protein 2 (RARRES2). It has been suggested to play a role in the metabolic syndrome. It is secreted as 143 residue prochemerin, which is largely expressed in the liver, WAT, skin, pancreas and kidneys. It is also the main form in the plasma. The level of active chemerin is negligible in basal conditions [15]. Various isoforms of chemerin have been detected in different tissues and fluids, including plasma, synovial fluid, muscles, liver

and ovary. Three isoforms have been identified, chemerinS, chemerinF and chemerinK and three G-protein coupled receptors have been detected, CMKLR1 (chemokine-like receptor1), GPR1 (G protein-coupled receptor 1) and CCRL2 (chemokine receptor like 2). CMKLR1 is coupled with the Gi/o proteins and inhibits the cyclic adenosine 3′,5′-monophosphate (cAMP) signaling pathway, promoting Ca^{2+} mobilization and extracellular signal–regulated kinases (ERK1/2) activation. The CMKLR1 sequence is closely related to GPR1 and activates the same pathways, while the role of CCRL2 remains unclear. Chemerin binding does not promote any signaling pathway and does not induce CCRL2 internalization [15]. Chemerin was reported to regulate adipogenesis, adipocytes differentiation, insulin secretion, inflammation and blood pressure. Obesity and type 2-diabetes are associated to high levels of chemerin [15].

2.4. Resistin

Resistin is a cysteine-rich, secretory protein, also known as adipocyte secreted factor (ADSF), belongs to the family of Found in Inflammatory Zone (FIZZ) proteins [16]. Resistin is produced by the adipocytes in mice whereas it is predominantly expressed in macrophages in humans. Human resistin is a 12.5 kDa cysteine-rich peptide with a mature sequence consisting of 108 amino acids, while the rat and mouse resistin has 114 amino acids. The comparison of the amino acid sequences of bovine resistin with that of humans, pigs, rats and mice showed 73, 80, 58 and 57% identity, respectively [17]. The resistin concentration in human plasma and follicular fluid ranges from 5 to 50 ng/ml [18], while in the follicular fluid in pigs it is around 0.323 ng/μg protein, depending on the stage of the estrous cycle [19]. Recent reports have suggested potential receptors for resistin, such as an isoform of decorin (DCN), mouse receptor tyrosine kinase-like orphan receptor 1 (ROR1), toll-like receptor 4 (TLR4) or adenylyl cyclase-associated protein 1 (CAP1) [5]. Furthermore, it is well known that resistin activates signaling pathways in different tissues like the phosphatidyl inositol 3′ kinase/ protein kinase B (Akt), mitogen-activated protein kinases (MAPK) (ERK1/2 and p38), signal transducer and activator of transcription 3 (Stat-3) and PPAR type gamma (PPARγ). Several studies have identified positive correlations between resistin levels and the pathogenesis of obesity, adipogenesis and insulin resistance [16].

2.5. Vaspin

Vaspin, a member of the serine protease inhibitor family, has been identified in the visceral adipose tissue of Otsuka Long-Evans Tokushima fatty rats at an age when body weight and hyperinsulinemia peaked [20]. This adipokine was initially observed in mature adipocytes from epididymal, retroperitoneal, mesenteric and subcutaneous abdominal WAT from 30-week-old Otsuka Long-Evans Tokushima fatty (OLETF) rats [20]. Literature data indicates the involvement of vaspin in the development of obesity, insulin resistance or pathogenesis of the body's inflammatory reactions [21]. Vaspin receptors remain unknown, but some data suggest that vaspin acts as a ligand for the cell-surface GRP78/voltage-dependent anion channel complex [22]. The vaspin gene was expressed in new born and adult bovines; in new born animals, vaspin is highly expressed in the heart, small intestine, skeletal muscle and fat, whereas in adults, it is expressed in the heart, liver, lungs and skeletal muscles. Vaspin increased the Akt phosphorylation protein and decreased the nuclear factor NF-κB level in pancreatic β cell and in cultured endothelial cells [23].

2.6. Visfatin

Visfatin was first discovered as a growth factor (PBEF) and then as a type II nicotinamide phosphoribosyltransferases (NAMPT). Visfatin structure showed a dimer organization separated by an active site. The visfatin gene is highly conserved among different species, such as mice, rats, humans and fish. The expression has been studied in many organisms and the porcine visfatin is ubiquitously expressed in tissues [24]. In a canine model, visfatin protein is expressed in various tissues like the liver, heart, brain, lungs and muscle [25]. In cows, mammary epithelial cells express visfatin [26]. In chickens, the full-length cDNA of visfatin gene has been cloned and sequenced. In the latter species, visfatin is highly expressed in muscles and it is involved in muscle growth [27]. Because of this characteristic, visfatin is also called myokine. No receptor has yet been identified for visfatin, but it is able to activate the insulin receptor. Besides muscle growth, visfatin is also involved in glucose and fatty acids metabolism and in reproductive functions. Moreover, in pancreatic β-cells, visfatin increases the glucose-stimulated insulin secretion.

3. Reproduction in domestic animals

The ovary, by producing steroid hormones, has a key role in female reproduction. It ensures follicles growth and the timely release of fertilizable oocytes essential for pregnancy, by directing feedback mechanisms on the hypothalamus and pituitary. Disruption of this finely controlled network can lead to many clinical syndromes including premature ovarian failure, ovarian hyperstimulation syndrome, ovulation defects, poor oocyte quality and cancer. Generally, in domestic animals, the oestrus cycle is composed of four phases: proestrus, estrus, metestrus and diestrus [28]. Each of these stages is a subdivision of the follicular and luteal phases of the cycle. For example, the follicular phase includes proestrus and estrus, while the luteal phase includes metestrus and diestrus [28]. Proestrus is characterized by a significant rise in estradiol (E2) produced by developing follicles. When E2 reaches a certain level, the female enters behavioral estrus and then ovulates. Following ovulation, cells of the follicles are transformed into corpus luteum (CL) cells during metestrus. Next, diestrus is characterized by a fully functional CL and high progesterone (P4) concentration [28].

3.1. Cows

The initiation of puberty is achieved when heifers reach 40 to 55% of their adult weight, which is strongly influenced by the nutritional level received during the prepubertal period [28]. At traditional high levels of energy intake, the onset of puberty may range from 7 to 10 months of age, 6 to 9 months earlier than for heifers of the same breed fed with a low energy nutritional intake [28]. Before the onset of puberty, the frequency of luteinizing hormone (LH) peaks increases leading to a brief P4 priming followed by the pubertal preovulatory surge of LH associated with behavioral estrus. The ovaries of heifers contain growing follicles that release steroidogenic hormones: P4 and E2 are regulated by the endocrine retro-control of the hypothalamus-pituitary-ovarian axis. In mature cows, waves of follicular oestrogens from granulosa cells (GC) activate gonadotropin releasing hormone (GnRH) and LH pulsation

that induce a positive feedback on E2 secretion until it reaches the preovulatory peak of LH and triggers ovulation. In cattle, the wave-like pattern of follicle development is very well characterized with most estrous cycles comprising two or three waves [28] (**Figure 2**). In the absence of fertilization, the endometrium secretes prostaglandins and CL regress and become atretic. Conversely, when fertilization occurs, CL cells continue to secrete P4 that stop the progression of a new estrous cycle [28]. In bovines, the gestation lasts 9 months. In breeding, farmers and researchers often resort to hormonal stimulation to synchronize ovulation and artificial insemination (AI). Over generations, the genetic selection and the high management for milk production have led to drastic negative energy balance and decline of fertility [28]. "Fertil+" cows had a significantly higher success rate at the first AI than "Fertil−" cows, without variations of ovarian dynamics. Traditionally, a decrease in the pregnancy rate at first service and the increase in the calving intervals and calving to first AI interval are associated with prolonged postpartum anestrus (the first ovulation is delayed), abnormal estrous cycles or follicular cyst formation [28].

3.2. Pigs

Pigs reach puberty close to the 7th month of postnatal life (**Table 1**); animals with higher fat mobilization reach sexual maturity faster and have more estrous cycles compared to animals with lower fat mobilization [29, 30]. The ovary begins to form at the 24-26th week of prenatal life, and the first ovarian follicles appear about 70 days after fertilization. Primary ovarian follicles comprise an oocyte surrounded by a single flattened layer of GC outlined by a basement membrane. Secondary follicles have a higher volume and number of GC and follicular

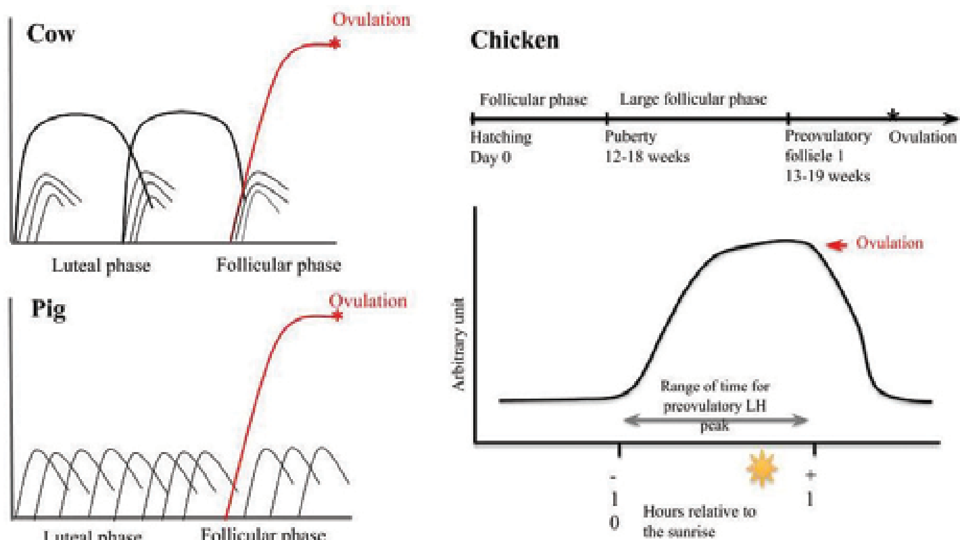

Figure 2. Schematic presentation of the pattern of follicle development during estrous cycles in cattle and pigs. In chickens, the pre-ovulatory release of LH can only be initiated between the beginning of the night and 1 hour after the sunrise. In addition, the ovulation is induced by a P4 peak under the control of LH.

Animal	Onset of puberty	Age at first Service	Estrous Cycle	Estrus	Gestation
Cows	1–2 yr	1–2 yr	21 d	18 h	282 d
Pigs	7 mo	8–10 mo	21 d	2 d	114 d
Chickens	12–18 wk	21 wk	24 h	no	21d

Table 1. Average ages or times for some reproductive parameters selected by species [28].

sheath is formed. Antral follicles formation depends on the follicular fluid accumulation, GC proliferation and theca cells (TC) differentiation. Preovulatory follicles are the final stage of the follicle before ovulation [29]. Swines do not show the wave-like pattern of follicle development that is so typical in ruminant species [28, 30]. While there is a coordinate development of follicles at the beginning of the luteal phase, there is continuous growth and atresia of ovarian cells during the rest of the luteal phase without evidence of dominant follicles or follicular waves (**Figure 3**) [29]. The outer wall of the ovarian follicle is the TC externa and the inner called TC interna. The TC externa implements an isolating function, while the TC interna function is secretory. The internal wall of the antral follicle is composed of GC, separated from the TC interna by the basement membrane. The last structure distinguished in the follicle is the oocyte, surrounded by the zona pellucida and corona radiata of granular cells [28]. The ovarian maturation is possible owing to the increasing number of follicle-stimulating

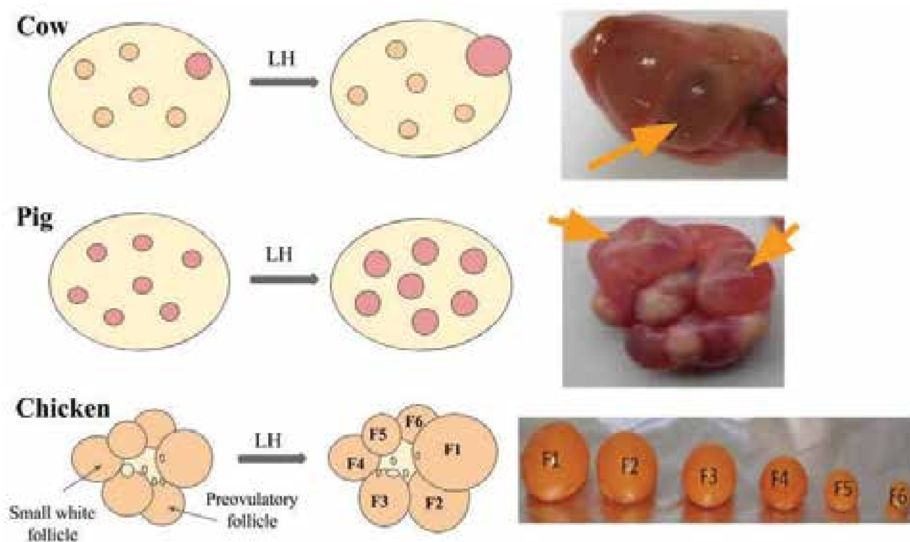

Figure 3. Ovarian follicles development and morphology. In cows, an early event in follicular selection is the acquisition of LH receptors in the GC of the presumptive dominant follicle. In pigs, multiple follicles acquire LH receptors in the GC at a less mature stage of follicular development; more preovulatory follicles are selected in pigs because more follicles have the capacity to survive during the transition from FSH to LH dependence. In reproductively active chickens, the ovarian cortex contains a hierarchy of follicles in all stages of development, from primordial follicles to large, yellow, yolk-filled pre-ovulatory follicles. AT gross inspection, the large follicles protrude from the surface of the ovary on short stalks while small follicles bulge from the surface.

hormone (FSH) receptors, which activates the aromatase complex involved in converting testosterone into E2. The secretion of E2, in turn, promotes the formation of the LH receptor in GC [29]. Pigs' estrous cycle lasts 21 days and is distinguished at 4 periods: proestrus, oestrus, metaestrus and diestrus. In the ovarian cycle two phases are distinguished: follicular (7 days), in which ovarian follicles are recruited and matured, and the luteal phase (14 days). The phases are separated by ovulation. The luteal phase starts in the first day after ovulation, and continues until day 15 of the estrous cycle. The early follicular phase starts on day 16–17 [29]. During the oestrus cycle different hormonal profiles are observed: E2 and inhibin gradually increase after luteolysis, and peak in the periovulatory period, provoking the LH surge. LH influences the final oocyte maturation, ovulation and CL formation. In the luteal phase, CL takes over the production of steroid hormones and secretes P4, which prepares the endometrium for embryo implantation and maintaining pregnancy for the first 12 weeks [28]. Porcine CL remains insensitive to prostaglandin F2α up to day 12 of the cycle; prostaglandin F2α degrades the luteal capillaries and reduces P4 production, leading to CL death. When there is no fertilization, the CL changes to atretic CL [28, 30]. Luteolysis begins on the 13th day of the cycle when the endometrium releases F2α prostaglandins, P4 synthesis is inhibited and new follicles grow. The percentage of fertilizations ending in pregnancy in pigs is high and reaches to 90%. Pregnancy lasts 112–114 days, producing a litter of 8–12. CL is essential for maintenance of pregnancy in pigs [30].

3.3. Chickens

Firstly, in birds, the sexual chromosomes are different from mammals. The female is the heterogametic with the chromosomes Z and W, while the male is homogametic with two Z chromosomes [31]. Then, in almost all species of birds, only the left ovary is functional. In hens, it develops after 16 weeks of age. It is organized in a single hierarchy consisting approximately of 6 preovulatory follicles. F1 is the largest preovulatory follicle and F6, the most recently selected from the cohort [31] (**Figure 3**). In domestic hens, sexual maturation occurs at about 5 months of age and is closely linked to the photoperiod. The follicular growth is associated with the formation of the TC interna layer. The preovulatory follicles rapidly grow and can reach more than 40 mm in diameter. The process of follicular selection is based on the ability of the FSH receptor to initiate cell signaling via cyclic adenosine monophosphate specifically in the GC [31]. Like mammal species, the preovulatory surge of LH is the first stimulus for the germinal vesicle breakdown and for ovulation. In avian species, the LH peak precedes ovulation by 4–6 hours. The largest preovulatory follicle produces a greater amount of P4 during the LH surge. This production is related to high levels of STAR in the GC of the F1 follicle [31]. Contrary to mammal species, there is no apparent increase in circulating FSH corresponding to the LH peak. Following the ovulation, the steroid production decreases and the regression of the postovulatory follicle is related to apoptosis, inducing the production of immune cells and cytokines. In birds, TC expresses aromatase and synthetize estrogens from androgens precursors, which are localized in TC. However, P4, pregnenolone and androgen precursors are produced almost exclusively within the TC interna, while GC produces P4 from cholesterol. These cells are able to convert P4 to testosterone but not to oestrogens [31]. Moreover, the hen oviduct is able to store sperm for a prolonged period in specialized sperm

storage tubules (SST), located in the utero-vaginal junction of the oviduct. Only the “normal” spermatozoa enter the SST indicating that some process of selection occurs [31].

4. Effect of adipokines on the ovarian function in domestic animals

Based on the available data, it is known that adipokines and their receptors are expressed in the ovarian cells and can modulate ovarian physiology, especially steroidogenesis, cell proliferation, apoptosis and/or oocyte maturation in domestic animals like pigs, cows, chickens, goats, ewes and turkeys (**Table 2**).

4.1. Adiponectin

In cows, serum adiponectin decreases from 21 days before calving, reaches a nadir at calving, before rapidly decreasing upon advancement of lactation [32]. During the bovine oestrus cycle, serum adiponectin levels range between 27 and 32 μg/ml and were 1.6-fold lower in

Adipokines	Ovarian function in domestic animals		
	Cows	**Pigs**	**Chickens**
Adiponectin	↓ A4, LHR, CYP11A1, CYP17α1; ↑ IGF1-induced and basal proliferation; ↓ insulin-induced P4 and E2	Follicles: ↑ expression of genes associated with periovulatory remodeling; ↑E2; CL: ↓ P4 cells	↑ IGF1-induced P4 ↓ LH and FSH-induced P4
Apelin	↑ IGF1-induced P4 and cell proliferation; ↓ IGF1-induced oocyte maturation and P4 from cumulus cells through MAPK ERK1/2	Follicles: ↑ basal P4, E2; ↓ IGF1-, FSH-induced P4, E2; ↑ proliferation; CL: ↑ P4 and 3βHSD	nd
Chemerin	↓ P4 and E2 at basal and in response to IGF1 or FSH through CMKLR1; ↓ StAR, CYP19 and MAPK-ERK1/2	nd	nd
Resistin	↑ FSH and IGF1-induced E2; no effect on IGF1- or insulin-induced P4 and A4 production by Tc or P4 production by Gc of large follicles; ↑ IGF1-induced P4 and E2 in small follicles;	↑ basal P4, T and A4; ↓ gonadotropin- and IGF1-induced P4, T, A4 and E2 by inhibition of 3βHSD, 17βHSD and CYP19A1; ↓ apoptosis;	nd
Visfatin	↑ basal and IGF1-induced steroid secretion; ↑ StAR, 3βHSD, IGF1-R, MAPK ERK1/2	nd	↓ IGF1-induced P4

CL, Corpus luteum; IGF1, Insulin-like factor 1; FSH, Follicles stimulation hormone; P4, Progesterone; A4, Androstenedione; E2, Estradiol; IGF1, Insulin-like growth factor 1; CL, Corpus luteum; Tc, Theca cells; ↑, increased; ↓, decreased; nd, no data.

Table 2. Effect of adipokines on the ovarian physiology in domestic animals.

follicular fluid, but the concentrations in the serum and follicular fluid were not correlated [33]. The circulating levels of adiponectin were decreased when providing low energy diet to the cows, and were related to a delayed and abnormal luteal activity in lactating cows [34]. *In vitro*, adiponectin (3 μg/ml) decreases the production of androstenedione (A4) by TC in cows by reducing the expression of LH receptors and CYP11A1 (cytochrome P450, family 11, subfamily a, polypeptide 1) and CYP17α1 (cytochrome P450, family 17, subfamily a, polypeptide 1) enzymes mediated by both receptors AdipoR1 and AdipoR2. With a higher dose (10 μg/ml), adiponectin increases insulin like growth factor 1 (IGF1)-induced GC and basal TC proliferation [33]. Additionally, adiponectin decreases insulin-induced secretions of P4 and E2 by GC but has no effect on the nuclear maturation of oocytes or early embryo development [5].

In pigs, adiponectin and its receptors expressions are observed in the ovary and a decrease of the 250 kDa adiponectin isoform has been reported in both follicular fluid and serum [35]. Adiponectin system is also expressed in the endometrium, myometrium and conceptuses and is dependent on the stage of the pregnancy. The highest level of adiponectin is observed on days 15 to 16 of the pregnancy and on days 10 to 11 of the cycle on the endometrium. The highest expression of AdipoR1 and AdipoR2 is detected on days 10 to 11 in the endometrium and on days 12 to 13 in the myometrium [36], around the period of the maternal recognition of pregnancy. The *in vitro* studies on porcine uterine tissues showed that adiponectin affects the gene expression of key enzymes involved in the steroid synthesis (StAR, CYP11A1 and 3β-HSD), and influences P4 and A4 secretion and prostaglandin synthesis pathway in the porcine uterus [37]. Using GC collected from medium-sized (3–5 mm) follicles of prepubertal gilts ovaries, Ledoux et al. [38] showed that adiponectin is present in the porcine follicular fluid at concentrations similar to those found in serum. These authors demonstrated that adiponectin at physiologically relevant levels (10–25 μg/ml), induces the expression of genes like cyclooxygenase-2, prostaglandin E synthase, and vascular endothelial growth factor genes, which are associated with periovulatory remodeling of the ovarian follicle [38]. The expression of adiponectin is significantly higher in porcine CL during the luteal phase compared with TC isolated on days 17–19 of the cycle [39]. Adiponectin at doses between 1 and 10 μg/ml significantly decreased P4 secretion by CL cells *in vitro*, and increased E2 secretion by GC, but had no effect on T secretion by TC [39].

In chickens, the adiponectin gene was found in the ovary, and it was 10- to 30-fold higher expressed in TC than in GC from each of the follicles (F1-F4) [40]. For the receptors, the AdipoR1 mRNA level is two-fold lower in TC than GC, while the AdipoR2 expression remained stable in both ovarian cells and during follicular development. Adiponectin exerts an autocrine or paracrine effect on ovarian steroidogenesis. In F2 and F3/4 follicles, adiponectin increased IGF1-induced P4 production. In F3/4 follicles, it decreases P4 production in response to LH and FSH [40].

In turkeys, the adiponectin plasma profile is significantly lower at the end compared to the beginning of the laying period. Furthermore, the hexameric form is reduced by three-fold at the end in comparison to the start of this period [41].

4.2. Apelin

In bovine species, it has been shown that apelin and apelin receptor (APJ) mRNAs are expressed in the bovine follicle and CL [42]. In the CL, apelin expression increases during the

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early luteal stages and decreases at the end of the luteal phase and during CL regression [42]. Apelin expression is only higher during pregnancy [42]. Thus, the apelin/APJ system could be involved in vascular establishment, maturation and maintenance of the CL during the estrous cycle. In TC, the expression of apelin is increased by E2 (5–180 ng/ml) and LH (100 ng/ml), while the expression of APJ is only increased by LH. In GC, the expression of APJ is increased by P4 (10 ng/ml) and by FSH (100 ng/ml) [43]. In mature bovine follicles, the apelin/APJ is thought to play a crucial role during follicle selection and dominance. During an *in vitro* study, apelin (10^{-9} M) increases IGF1-induced P4 secretion and cell proliferation in bovine luteinizing GC, whereas it inhibits IGF1-induced oocyte maturation and P4 secretion from cumulus cells through the regulation of extracellular signal-regulated kinases MAPK ERK1/2 phosphorylation [42–44].

In porcine, apelin concentration in the follicular fluid and its expression increases with ovarian follicular growth. Immunohistochemistry revealed the positive higher staining for apelin in membranes of GC, than TC [45]. Apelin was found to increase secretion and 3βHSD and CYP19 expression in co-culture of GC and TC as well as cell proliferation, while decrease IGF1- and FSH-induced steroidogenesis [45]. As a molecular mechanism of these observation, authors showed that AMPKα was involved in the action of apelin on P4 production but MAPK/ERK, AMPKα and Akt/PI3 mediated the proliferative effect of apelin [45]. In an *in vitro* experiment involving CL cells, apelin also increased P4 secretion by the activity of 3βHSD [46]. Expression of mRNA apelin in CL were similar in the early and middle CL, and less in late CL. Apelin has been shown in the cytoplasm of both, small and large luteal cells by immunohistochemistry [46].

4.3. Chemerin

In bovine species, chemerin reduces *in vitro* P4 and E2 secretion at basal levels, in response to IGF1 or FSH through its main receptor, CMKLR1 [47]. Chemerin also reduces cholesterol content, the protein quantity of the cholesterol carrier StAR, the protein CYP19 and the level of phosphorylation of the MAPK-ERK1/2 in the presence or absence of IGF1 and FSH [47]. In the bovine cumulus-oocyte complex, chemerin arrests the majority of oocytes at the GV stage *in vitro* which is associated with a reduction in P4 secretion by the cumulus cells and the phosphorylation of the MAPK-ERK1/2 signaling pathway in the oocyte and cumulus cells [47].

In chickens, chemerin and its receptors are higher in TC than in GC in both preovulatory follicle 1, follicle 3 and 4 [48]. Chemerin is negatively correlated with the rate of hatchability. In contrast, the weight of the preovulatory follicle F1 is positively correlated with the chemerin expression in GC. Finally, the P4 production in GC is negatively correlated with the chemerin expression in TC. In hens, the diet has a significant impact on the reproductive function [48]. Thus, restricted fed hens expressed lower chemerin mRNA expression in TC from preovulatory follicles 1 and 3 than ad libitum fed hens. A fish oil supplementation increases the mRNA level of CMKLR1 in TC of preovulatory follicle F1 but decreases it in TC of preovulatory follicle F3 [48]. The chemerin gene sequence is similar at 81% between chickens and turkeys. In turkeys, chemerin concentration in plasma decreases at the end of the laying and is negatively correlated with phospholipids, triglycerides and cholesterol levels during this period [41]. The role of chemerin in the different mechanisms of the reproduction remains to be considered.

4.4. Resistin

Resistin is widely expressed in differently sized bovine follicles (small <6 mm and large >6 mm), where it was demonstrated in oocytes, cumulus, TC and GC, as well as in the CL [49]. In cattle, resistin at 30 ng/ml weakly stimulated FSH plus IGF1-induced E2 production but had no effect on IGF1- or insulin-induced P4 or A4 production by TC or P4 production by GC of large follicles [50]. However, in GC from small follicles, resistin attenuated the stimulatory effect of IGF1 on P4 and E2 secretion. Moreover, it has been documented that, resistin stimulated Akt and p38-MAPK phosphorylation in bovine and rat GC, ERK1/2-MAPK phosphorylation in rats and had the opposite effect on the AMPK pathway [49].

In pig ovaries, resistin levels and expression varies with the stage of cycle. Differences in the resistin expression and concentration in follicular fluid collected from small, medium, and large follicles have also been reported [19]. Interestingly, in contrast to prepubertal animals, resistin expression and concentration in adult estrous cycling pigs was independent of follicular size and/or development [5]. Moreover, several factors can influence ovarian resistin expression, which has shown to increase with gonadotropin and steroid hormones and decrease with IGF1 and rosiglitazone (a PPARγ-selective agonist) [5]. Resistin affected the ovarian steroidogenesis, decreasing gonadotropin- and IGF1-induced steroid hormone secretion by the inhibition of 3βHSD, 17βHSD and CYP19A1 protein expression [5]. In ovarian follicles resistin by direct effects on both death receptor- and mitochondria-mediated apoptosis protein, was described as an anti-apoptotic factor [5]. It has been proposed the activation of several signal transduction pathways, such MAPK/ERK1/2, Janus-activated kinases (JAK)/STAT and phosphatidylinositol 3-kinase (PI3K) as a molecular mechanism of resistin action on cell survival [5]. These results suggest the involvement of resistin in ovarian apoptosis regulation and could regulate follicular development or atresia.

4.5. Visfatin

In cumulus cells from "Fertil−" cows, visfatin mRNA expression was lower compared to "Fertil+" cows, especially after *in vitro* maturation [51]. During *in vitro* experiments, visfatin improves basal and IGF1-induced steroidogenesis, probably through increasing the protein level of StAR, the 3βHSD activity and the phosphorylation levels of IGF1-receptor and MAPK ERK1/2 [52].

In chickens, visfatin mRNA is higher in GC than in TC, and it regulates steroidogenesis in ovarian cells. During the folliculogenesis, the expression in TC decreases whereas it remains stable in GC. It inhibits IGF1-induced P4 production in GC. Moreover, the plasma level is significantly lower in adult hens than in juveniles [53]. In turkeys, visfatin mRNA level is higher in TC than in GC in follicles F1, F3 and F4, like chemerin and adiponectin expression. Moreover, visfatin concentrations in plasma decrease and are negatively correlated to plasma glucose during the laying period [41].

4.6. Vaspin

Vaspin expression and roles on female reproduction remain unknown. In porcine ovarian follicles, our preliminary results evidenced mRNA and protein expression of vaspin, whose

expression was decreased during ovarian follicle development. We also observed that protein expression of vaspin was lower in large follicles of low-fat mobilization pigs (*Large White*) than in high fat mobilization pigs (*Meishan*), but this data should be confirmed.

5. Conclusion

Taken together, this chapter summarizes the expression and direct role of different adipokines in ovarian follicle cells. These observations clearly documented that adiponectin, apelin, chemerin, resistin, visfatin and vaspin are expressed on mRNA and protein level in the ovarian GC, TC and CL, suggesting that the ovary is a target organ for adipokines production and secretion. It is interesting that several *in vitro* experiments have documented that these peptides can regulate the ovarian physiology such as the steroid hormone production cell proliferation, apoptosis and oocyte maturation in different domestic animals like pigs, cows, chickens or turkeys, and should be considered a newly identified regulator of female reproduction.

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Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations

WAT	white adipose tissue
HMW	high-molecular weight
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
PPAR	proliferator-activated receptor
APJ	apelin receptor
GH	growth hormone
TNF-α	tumor necrosis factor

BMI	body mass index
TIG2	termed tazarotene-induced gene 2 protein
RARRES	retinoic acid receptor responder protein 2
CMKLR1	chemokine-like receptor 1
GPR1	G protein-coupled receptor 1
CCRL2	chemokine receptor like 2
cAMP	cyclic adenosine 3′,5′-monophosphate
ERK1/2	extracellular signal–regulated kinases
ADSF	adipocyte secreted factor
FIZZ	inflammatory zones
DCN	decorin
ROR1	tyrosine kinase-like orphan receptor 1
TLR4	toll-like receptor 4
CAP1	adenylyl cyclase-associated protein 1
Akt	protein kinase B
MAPK	mitogen-activated protein kinases
Stat-3	signal transducer and activator of transcription 3
OLETF	Otsuka Long-Evans Tokushima fatty
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PBEF	pre b cells enhancing factor
NAMPT	type II nicotinamide phosphoribosyltransferases
E2	estradiol
CL	corpus luteum
P4	progesteron
LH	luteinizing hormone
GC	granulosa cells
GnRH	gonadotropin releasing hormone
AI	artificial insemination
FSH	follicle-stimulating hormone
TC	theca cells

StAR	steroidogenic acute regulatory protein
SST	sperm storage tubules
A4	androstenedione
LHR	the luteinizing hormone receptor
CYP11A1	cytochrome P450, family 11, subfamily a, polypeptide 1
CYP17α1	cytochrome P450, family 17, subfamily a, polypeptide 1
IGF-I	insulin-like growth factor-1
3βHSD	3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase
17βHSD	17β-Hydroxysteroid dehydrogenases
IGFi-R	insulin-like growth factor-1 receptor
AMPKα	5′AMP-activated protein kinase
GV	germinal vesicle
PI3K	phosphatidylinositol 3-kinase
JAK	Janus-activated kinases

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Chapter 4

Application of Some Proteome Analysis Techniques in Animal Reproduction

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Additional information is available at the end of the chapter

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Abstract

This chapter focuses on the application of proteome analysis techniques to animal reproduction and provides general information on one-dimensional (1D) and two-dimensional (2D) electrophoresis, chromatographic methods, and mass spectrometer (MS), widely used in proteomics studies. Proteome consists of an entire complement of proteins expressed by a cell, tissue, or organism. Proteomics reveals functions of proteins encoded in the genome. These functions include posttranslational modifications (PTMs) and alterations in the protein synthesis. Animal reproduction takes a key role in livestock, and increasing the reproduction rate in flocks plays an important role in livestock management. Studying the proteins related to reproduction could guide on how to increase fertility. Recent studies addressed the proteome constitution of both male and female reproductive system. Follicular fluid, endometrium, and ovary proteins were analyzed in females by proteomics study, while in males, sperm proteomics was more focused. Information obtained on this issue is also beneficial for the development of reproductive technologies such as *in vitro* fertilization and embryo transfer. Strategies to increase fertility in animals can be revealed by proteomic studies, and a more profound knowledge on proteomics may become helpful to develop and enhance the efficiency of reproductive technologies.

Keywords: animal reproduction, protein screening, chromatography, electrophoresis, mass spectrometer, proteomics

1. Introduction

Reproduction is crucial for the existence and survival of a species. Also, being successful in animal farming depends on the reproductive efficiency of the individuals. There are three

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main aims to the livestock reproductive efficiency: maintaining of herd size, providing the productivity and effectiveness in selection or breeding, and culling. Fertility is influenced mainly by genotype and environment. Besides, anatomical fitness and hormones have an important impact on fertility. In livestock, various reproductive technologies have been developed to escape infertility. Artificial insemination, embryo transfer, *in vitro* fertilization, and cloning are some of them. The development and enhancement of the techniques have benefit from the understanding of reproduction system in a proteomic basis.

Proteomics was first described by Wilkins and Williams in the mid-1990s [1]. Very simply, proteomics is a branch of biotechnology that involves gene-level protein expression studies. Two definitions of proteomics have occurred; first is restricting the large-scale analysis of gene products to studies involving only proteins, and second is combining protein studies with analyses that have a genetic readout, such as mRNA analysis and genomics [2]. However, the goal of proteomics is to obtain a more global and integrated view of biology by studying protein and genome. Furthermore, proteomics can provide exciting new opportunities to dramatically advance knowledge in a system perspective view of biology. Moreover, it also enables identifying new drug targets, leads to the development of superior diagnostics and therapeutics, and correlates biological pathways and molecular mechanisms in the disease [1]. Proteomics approaches can be used for protein profiling, comparative expression analysis of two or more proteins, the localization and identification of posttranslational modifications, and the determination of protein-protein interactions [3].

Crops not consumed in human diets are utilized by farm animals and turn to good quality products such as meat, milk, and others. Hence, livestock provides the quality source of protein for the human consumption, contributing to a balanced diet for the majority of the populations.

However, proteomics has been partially omitted from farm animal research [4] until recently. Proteins in animals were first studied in 1975 on guinea pigs and mice [5, 6]. In twentieth century, the studies were conducted with laboratory animals, mainly for the understanding of human protein metabolism [7]. The usage of proteomics in farm animals is more related with meat and milk industry. However, proteomics takes part of the clinical diagnostics of mastitis, intestinal and respiratory diseases, or parasites and helps to understand their pathogenesis [8]. Proteomics can get different names whereby according to usage in research areas: nutriproteomics, immunoproteomics, histoproteomics, meat proteomics, serum proteomics, etc. Taking from the names, it seems that proteomics has a wide variety of subject areas or matrices in animal science.

1.1. Proteomics in the reproduction field

At a molecular level, reproductive processes are complex phenomena that occur by interactions of many proteins. Although reproduction is understood at the molecular level, it is far from being understood in what way the reproductive phenotype and behavior relate to its

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molecular foundations. It has been conjectured that female and male reproductive proteins can be identified by proteomics to ease the understanding of this mechanism. Different works have been developed using a proteomic approach in the reproduction field.

For example, the litter size is important parameter of sow productivity. It is emphasized that placental efficiency and litter size were highly correlated. It has further been shown that the placental proteins profiling as biomarkers could be used to estimate porcine litter size. Six proteins were identified in pigs with small size, contrasting to the 13 produced by pigs with large litter size [9]. Aminopeptidase (PSA, 70 kDa) and retinol-binding protein 4 (RBP4, 23 kDa) were the dominant proteins in large litter size group, as confirmed by western blot. In addition, PSA was determined as two different molecular weights (70 and 100 kDa) in both groups. PSA with 100 kDa was more expressed in the small litter size group [9].

Another example could relate to the understanding of hyperprolificacy in sheep. Using a proteomic approach, it was determined that a low level of ribosome-related proteins in the follicular fluid with isobaric tag for relative and absolute quantification-based proteomics was associated to high ovulation rate in Han sheep [10]. In addition, the follicular fluid provides a microenvironment for nuclear and cytoplasmic maturation of the oocytes. For *in vitro* fertilization, it is important to collect as many oocytes as possible. The oocyte selection in an *in vitro* fertilization program relies mainly on morphological criteria [11]. Precisely at this point, the proteome of the ovarian follicular fluid may provide information on the potential fertility in all animal species. The amount of serotransferrin, zinc-alpha-2-glycoprotein-like, complement factor B, and complement protein C3 changes in the follicular fluid according to the oocyte size [12]. The knowledge on the protein constitution of the follicular fluid could be useful to optimize the success of *in vitro* embryo production [12].

Similarly, changes in follicular fluid proteomics have been revealed in less-fertile dairy cows [13]. As known, the low fertility is common in high-yielding dairy cows, leading to important economic losses mainly on regards to the number of the inseminations, the increased maintenance costs, even treatment costs, and a lower number of calves. A total of 219 proteins were identified in the follicular fluid of preovulatory follicles in dairy cows, 26 of them uncharacterized [13]. Alpha-1-antiproteinase, metalloproteinase inhibitor 2, inter-alpha-trypsin inhibitor heavy chain H1, basement membrane-specific heparin sulfate proteoglycan core protein, complement component C8 alpha chain, collagen alpha-2(I) chain, prothrombin, alpha-S1-casein, interleukin-1 receptor accessory protein, and one uncharacterized protein (its gene named LOC781004) were differentially expressed in low-fertile dairy cows compared to controls [13]. Other studies identified 143 proteins in Holstein cows' follicular fluid during follicle development [14] and 363 proteins in buffalos follicular fluid [15], while other studies identified 38 and 463 protein spots in the female dog [16] and the mare [17].

Success in pregnancy depends on a good implantation and the uterus fitness. Although pigs have high fertility ratio, early embryonic losses are relatively common in this species. In this context, identifying the endometrium proteins can be significant. Several proteins were identified from sow endometrium, which play a role in the preparation of the endometrium for implantation [18]. In addition, some changes reported in the protein constitution have been

associated to the action of both maternal and embryonic hormones in the endometrium, during the embryo-maternal interaction, a crucial event for fertility [19].

Overall, in humans, 50% of the infertility causes are attributed to men. Male infertility is primarily caused by sperm dysfunction [20]. Biochemical fitness, DNA fragmentation, sperm abnormalities, and immunological problems are at the origin of this problem. Many studies, conducted on ejaculated semen, showed that the conventional methods have limited capacity to collect information on spermatogenesis and epididymal maturation. Many proteins were characterized during sperm epididymal maturation [21, 22]. Knowledge on the changes in the content and the location of these proteins during the sperm maturation process is critical for understanding spermatogenesis as well as fertility. As it is known, the spermatozoa leave the seminiferous tubules for posttesticular maturation with scarce content in cytosolic organelles and a complete loss of transcriptional ability [20]. In human, it has been shown that 35% of sperm-located proteins are of testicular origin and 48% of epididymis, while 17% are common to both organs [23]. Besides, sperm proteomic may also be useful in the field of *in vitro* fertilization. Different protein content was identified between male patients who experienced a failure in an *in vitro* fertilization procedure and healthy males [24]. Moreover, sperm proteomic also allows identifying the cryopreservation ability of semen within species. For instance, in boars, sperm membrane proteins can be used as markers for semen cryopreservation [25].

There are some limitations as well as advantages in the proteomics analysis. Limitations in proteomic generally depend on the characteristics of the tissue, fluid, or body system to be studied. Low-abundance, hydrophobic, and basic proteins show certain limitations in proteomics [26]. A good knowledge of quantities and properties of reproductive system proteins can increase the success of proteomic analysis and in determining the right instruments to be used in the analysis.

2. 1D and 2D electrophoresis

The studies on the separation of proteins are based on a history of about 250 years. The 1960s and 1970s are the most popular years for protein and enzyme studies and for the development of separation techniques. Since 1989, the interest in protein purification has increased considerably due to efforts to search for genes. It is often stated that the twenty-second century will be important for the properties of genes and corresponding proteins that are discovered and for understanding the functions of the organism [27, 28].

Proteins are macromolecules that combine a large number of amino acids. An amino acid is composed of a group of positively charged amino (NH_3) and negatively charged carboxyl (COOH); the charged components in the formation act on the net charge of a protein. According to the media, protein net charge can sometimes be positive and sometimes negative (amphoteric). Due to this basic feature of proteins, they can be separated in electrophoretic environments and their biological characterization can be facilitated. However, there is a certain pH value at which the net charge of each protein is "zero," which is called the isoelectric point (pI). Proteins are positively charged when their pI values are higher than

their pH values (pI > pH), and they are negatively charged when pH > pI. Proteins can also be separated according to their molecular weights. In this process, proteins are degraded into peptides and then charged minus with a detergent called sodium dodecyl sulfate (SDS). The peptides are then separated using a polyacrylamide gel. This method was first named as SDS-PAGE by Laemmli in his scientific article in 1970 [29]. Thus, SDS-PAGE can be considered as the ancestor of the novel gel-based separation techniques. In this method, two different polyacrylamide gels (stacking and separating) can be prepared and combined. This gel system is defined as discontinuous. With the aid of a comb that is placed on the "stacking gel" before it is gelatinized, the wells where the protein samples can be loaded into are formed—hence, it is also called "comb gel." This gel intents to sift and organize the high kilodaltoned proteins before they pass into the "separating gel." Many proteins can be examined in a separating gel with an acrylamide concentration of 10–12.5%. **Figure 1** shows an image of 1D electrophoresis according to Laemmli while the system is working. **Figure 2** shows the image of a 10% gel obtained from the quail serum after the 1D electrophoresis process was performed.

In 2D electrophoresis, proteins are first separated according to their PI and subsequently separated according to their molecular weights. With this method, up to 1000–2000 proteins can be separated at one time [30]. In the 1D stage, immobilized pH gradient (IPG) strips are used. One side of the IPG strips is covered with a polyacrylamide gel at different pH values (available in varying pH range and length), and the other side is covered by a plastic strip.

Figure 1. 1D electrophoresis according to Laemmli.

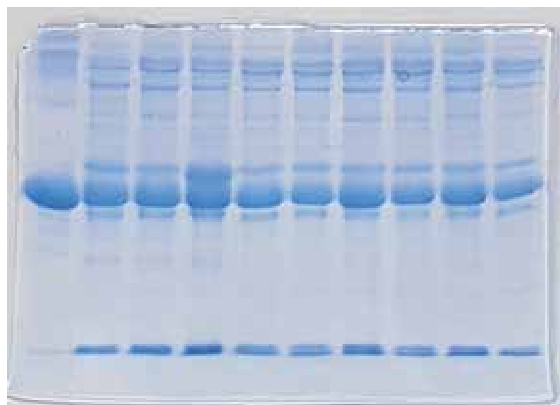

Figure 2. 10% gel image of quail serum after destaining in 1D electrophoresis.

IPG strips are placed over the protein samples (in a strip holder) prepared for analysis. The strip holder is then held in an electrical field (in 12,000 V for 1 h) until the net charge of the proteins is zero. After this step, strips are immersed in an equilibration solution, which is intended to the proper denaturation of proteins for an effective separation in 2D stage. The equilibration solution, usually prepared in a volume of 20 mL, is then divided into two equal portions: 10 mg dithiothreitol (DTT) is added to the first portion and 25 mg iodoacetamide (IAA) to the second. DTT is used for breaking disulfide bonds in the proteins, while IAA prevents the reformation of these broken disulfide bonds.

The 2D stage is performed in a vertical electrophoresis system. These systems generally consist of plates, casting stands, electrode buffer tanks, lids, and a power supply. The plates are assembled at regular spacing (0.75 or 1 mm) with the aid of spacers. In these plates, gel takes shape until gelatinization is complete. A casting stand is used to hold the plates in an upright position. Electric current is conducted in a buffer. The most commonly used electrode buffer is Tris-glycine (pH = 8.3). The electrical stream direction is from the cathode to the anode because proteins are negatively charged with SDS. The gel cassette placed in the buffer tank is filled with the electrode buffer and the system becomes ready by closing the lid.

IPG strips are placed horizontally on top of the prepared SDS-PAGE gel. A molecular weight marker is loaded on the first well of the gel to distinguish the proteins by their weight. However, unlike in 1D electrophoresis, the agarose gel pours over the stacking gel. The agarose gel prevents the development of an air gap between IPG strips and the separating gel. After separation, proteins are visualized by staining. Coomassie brilliant blue, silver stain, and fluorescent dyes are widely used. If fluorescent dyes are used, fluorescent imaging systems are required. Specialized software programs are used to measure the studied proteins. **Figure 3** shows a 2D gel image from the analysis of sheep caruncular endometrium. Mass spectrometry (MS) systems (MALDI-MS, ESI-MS, and LC-MS/MS) may also be used after the 2D stage for more detailed analysis and characterization of proteins. If MS will be used after 2D stage, the gel, SDS, and other residues such as bromophenol blue have to be removed. Trypsin, a serine protease enzyme, is needed for MS analysis. This enzyme separates proteins into appropriate lengths at which the MS device can measure [31]. 2D gel/MALDI-TOF MS may be a good choice for proteomic analysis as a starting point [26].

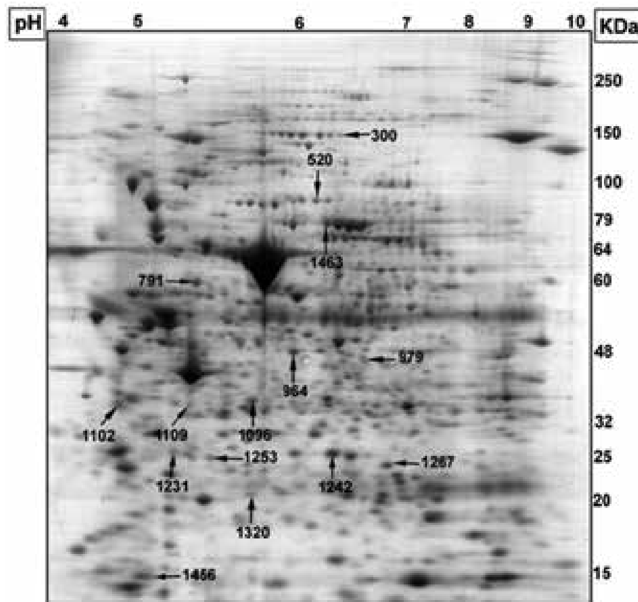

Figure 3. 2D gel image of sheep caruncular endometrium. Increasing pH from left to right and molecular weights of proteins from bottom to top [32].

3. Chromatography and MS

Chromatography is a widely used technique for separating the molecules. It is based on the principle that the molecules are separated when passing through a mobile phase from a stationary phase. While the mobile phase may be liquid or gas, the stationary phase is only solid or liquid. This technique is named according to the character of the mobile phase used. If the mobile phase is liquid, it is called "liquid chromatography (LC)" and if mobile phase is gas, it is called "gas chromatography (GC)." Apart from these, it is also classified according to the type of bedding (column or paper chromatography) and separation mechanisms (ion-exchange, partition, surface adsorption, and size exclusion chromatography) [33–35].

Proteins can be purified based on their size and shape, total charge, hydrophobic groups, and binding capacity with the stationary phase. These characteristics of the proteins allow the use of chromatographic methods, and column chromatography is the most frequent method for protein separation. There are many stationary/mobile phase combinations that can be employed. The chromatographic equipment needed depends upon the usage purpose. In chromatography systems, there must be established the conjunction with columns for the move through of stationary and mobile phase in the columns. Other accessories such as valves, fittings, fraction collectors, and autosamplers may be used to enhance the efficiency of output. For the data storage and evaluation, a computer console is usually combined with the system. **Figure 4** shows the scheme for an LC system.

Molecules are not normally charged, and mass spectrometers (MS) convert ions into charged ionic molecules. If the proteins get a polarity, the detailed analysis can be performed on them. The basic principle of mass spectrometry is based on a deflection of atoms toward different

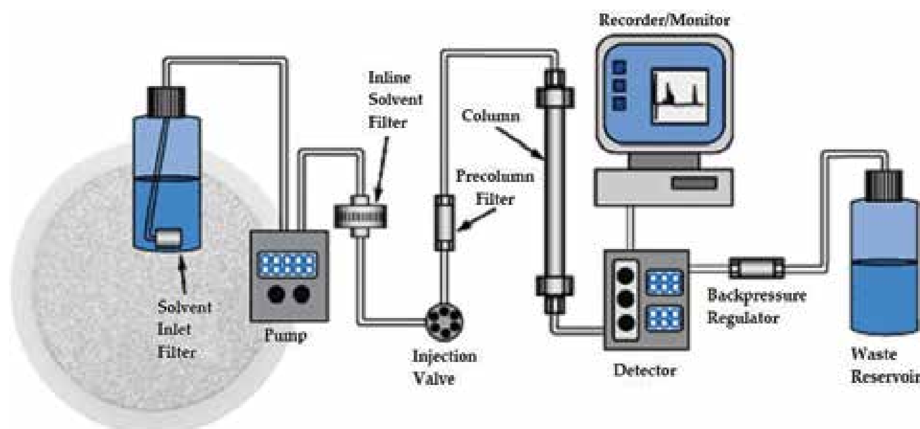

Figure 4. The schematic LC system [36].

orbits with the acceleration process in a magnetic electric field depending on their weights. Molecules separated in the LC method are more specific and quantitatively assessed with MS and the quality of LC's chromatographic property is increased. In MS, however, the particles separate not only according to their weight but also according to their charge. In the first stage of MS, the "ionization," particles are usually given a positive charge, and electrons are lost. The deflection velocity in the magnetic field will also change depending on the electrons lost by the particle. The particles that loss more electrons or are more positively charged will deflect more. It is important to take the air through vacuum at this stage, because the ionized particles may collide with air molecules impairing the obtention of a smooth flow. The particles are then accelerated to have the same kinetic energy (acceleration stage). Particles flowing at a certain velocity start to deflect according to their weight and charge (deflection stage). Finally, the particles are determined by the mass/charge ratio (m/z) to obtain data. For example, if a mass of a particle is 12, charge ratio is +1, m/z = 12, the mass of another particle is 24, but the charge ratio is +2, m/z = 12 again. **Figure 5** presents the general workflow scheme of MS.

Another available technique for protein identification and measurement is called tandem mass spectrometry or MS/MS. In this process, certain particles separated by m/z in MS1 are stimulated by the neutral gas (usually argon) contained in the collision chamber, and the particles are redispersed and transferred to MS2 and remeasured in m/z to m/z (**Figure 6**). Multiple MS cycles (MS/MS/MS or MS^n) can be developed for more precise studies [37].

However, particles with similar charge during the acceleration stage may show similar flow under the same kinetic energy, and therefore, particles are separated according to their flight times in the spectrometer. This technique is called time-of-flight mass spectrometry (TOFMS), and it is used for particle separation in many laboratories [40].

The quantitative analysis of biomolecules (proteins, peptides, nucleic acids, etc.) susceptible to breakage can be difficult in these techniques. At the same time, these techniques are labor and time-consuming, as well as costly. Matrix-assisted laser desorption/ionization (MALDI) technique has been developed to avoid measurement losses in the analysis of biomolecules susceptible to breakage (protein, peptide, nucleic acids, etc.). In this

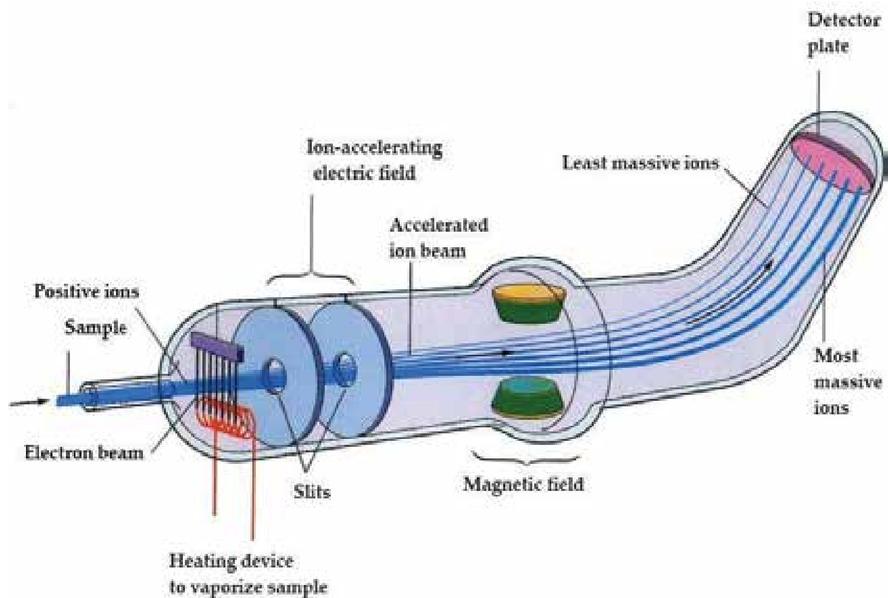

Figure 5. General workflow scheme of MS [38].

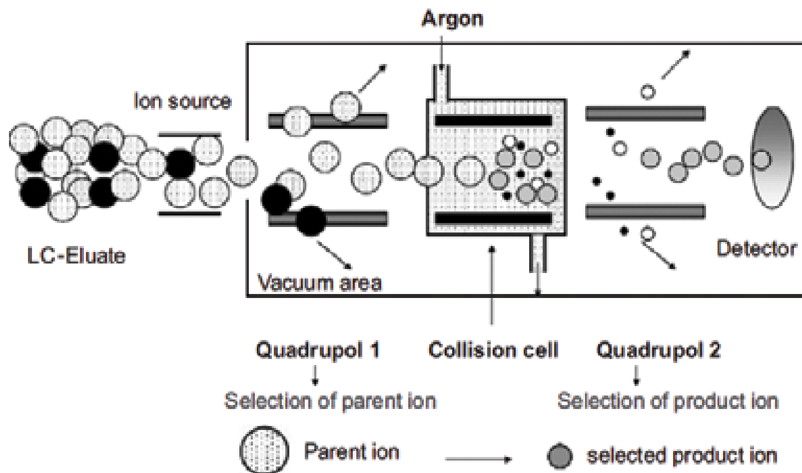

Figure 6. Principle of tandem mass spectrometry (MS/MS) [39].

technique, the first stage of MS, "ionization," is applied gently to prevent breakage. Briefly, in MALDI-MS, the sample is mixed with an ultraviolet light absorbing solution (the matrix, which is a compound of crystallized molecules). The liquid fraction in the matrix is then evaporated, and the matrix-sample mixture is crystallized. The sample is ionized via the matrix by sending the laser light to this crystallizing compound. The ions produced by the MALDI technique can also be measured with TOF (MALDI-TOF) to provide a high sensitivity and quantitative measurement. In a study that comparing MALDI-TOF MS with other automated methods provided by various companies [41], the error rates of the

Matrices	Peptides	Proteins	Nucleotides	Application
α-Cyano-4-hydroxycinnamic acid (CHCA)	✓			Smaller than 5000 Da
2,5-Dihydroxybenzoic acid (gentianic acid)	✓	✓		Low-weighted molecules
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid)		✓		Larger than 5000 Da
3-Hydroxypicolinic acid (HPA)			✓	Oligonucleotide detection

Table 1. Matrix types available for some biomolecules [44, 45].

Specifications	Quadrupole	Ion trap	TOF
Range (m/z)	3000	2000	∞
Resolution	Typical	Medium	Low
Adaptability	Poor adaptive for MALDI Well suited for ESI	Well suited for MS/MS	Suitable for MALDI

Table 2. Some specifications of quadrupole, ion trap, and TOF [46].

techniques (MALDI-TOF MS *vs.* VITEK GN+® in VITEK® 2, bioMérieux) were determined as 9.4 and 0.39%, respectively. At the same time, 6-h recovery and eightfold lower cost were achieved by MALDI-TOF compared to conventional methods (VITEK GN+® in VITEK® 2, bioMérieux) [41]. However, there are some factors that limit MALDI-TOF. It is difficult to combine this technique with other chromatograms, and it can work unstable with ions having a low m/z. In **Table 1**, matrix types for various biomolecules and some of their specifications are presented [41].

The electrospray ionization (ESI) is another "soft ionization" technique for break-sensitive biomolecules. This technique was first reported by Yamashita and Fenn in 1984 [42]. In this technique, there is a transition of ions aid by electrical energy into a gas chamber. In this system, movement of liquid occurs only by electrical power. The electrospray injection method is one of the most suitable techniques for providing uniform flow and particle size distribution in a low flow rate [43]. Some features of quadrupole, ion trap, and TOF, and their usability with other techniques are shown in **Table 2**.

4. Conclusions

Proteomics may become a helpful tool, allowing to better understand the mechanism underlying the biology of reproduction and fertility in both animals and men. The potential contributions of proteomics to the study of fertility can be considered as two directional: first, in the diagnosis of reproductive diseases and the second, in the identification of breeds and/or individuals with high fertility. Proteomics is also quite useful in the development of reproductive technologies and in supporting assisted reproduction. Proteomics has been slightly neglected in livestock research due to its high cost and labor. In the future, proteomic studies

in animal science will have an important place owing to the understanding of the benefits of proteomics in terms of animal health and production and the allocation of research funds by governments or funding organs.

Conflict of interest

The authors have no affiliations with or are not involved in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this chapter.

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Section 2

Hot Topics in Reproductive Medicine

Chapter 5

Subclinical Endometritis in Dairy Cattle

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Additional information is available at the end of the chapter

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Abstract

Subclinical endometritis is recognized as a cause of poor reproductive performance in dairy cows. Inflammation of the endometrium persisting after postpartum uterine involution has been related with prolonged calving-conception intervals and low fertility in dairy cows. The subclinical nature of this condition makes it necessary in the use of endometrial cytology or biopsy for diagnosing it. There are some controversies among authors in relation to the postpartum period from which a physiological endometrial inflammation should be considered a pathological subclinical endometritis. Therefore, depending on the sampling period after calving, different studies establish a different degree of polymorphonuclear leukocyte infiltration as cutoff point to diagnose subclinical endometritis. Controversies also exist regarding the pathogenesis of the disease and its consequences on the fertility of dairy cattle. The aim of this chapter was to review the current knowledge on this uterine pathology.

Keywords: dairy cow, reproduction, uterine pathology, inflammation, infertility

1. Introduction

One of the main factors affecting reproductive performance of dairy cattle is postpartum uterine disease. Metritis and endometritis have been associated with delays in restarting ovarian activity postpartum, prolonged intervals from calving to first service, increased number of days open, decreased conception rates, and increased culling rates [1–5]. Affected animals are easily identified when they show clinical signs indicative of uterine disease. Though

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symptoms of systemic illness are often absent, a purulent or mucopurulent vaginal discharge warrants further investigation, and therefore, clinical metritis and endometritis rarely remain undiagnosed.

Fourteen years ago, Kasimanickam et al. [6] found that many clinically normal postpartum cows had subclinical endometritis (SE). Those authors evaluated endometrial cytologies collected from 228 healthy cows at 21–33 days postpartum and related the cytological findings with the subsequent reproductive performance of cows. They used a receiver/response operating characteristic (ROC) curve to determine a threshold percentage of polymorphonuclear leukocytes (PMN%) in the cytological smears above which fertility was significantly reduced, and therefore, subclinical endometritis was diagnosed based on PMN% threshold. Since that pioneer work, many other studies have investigated the etiology, prevalence, and impact on reproduction of SE in dairy cows.

2. Etiopathogenesis

Subclinical endometritis is the inflammation of the endometrium without clinical signs and often without evidence of infection [7–9]. Alteration of the inflammatory response postpartum could be at the origin of this condition.

There is no doubt that uterine pathogens may negatively affect reproduction both by causing direct endometrial damage and by producing toxins [10, 11]. Bacterial endotoxins are known to have numerous effects on reproduction: (a) they may affect estradiol and progesterone secretion and alter follicular growth and the normal development of the corpus luteum [10–12], (b) may interfere with LH production and cause ovulation failure [13, 14], (c) may increase PGE_2 secretion and prolong the life span of corpus luteum [15], and (d) may induce embryo mortality [16].

In cows with metritis and clinical endometritis, recognized pathogens such as *E. coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, or *Prevotella* spp. are commonly isolated from the uterus [17]. In the case of SE, in contrast, several studies [7–9] showed that bacterial populations isolated from the uterus of cows diagnosed with SE did not differ from those of healthy cows. Prunner et al. [18] found that presence of *Trueperella pyogenes* in the uterus postpartum was a risk factor for development of clinical endometritis, but neither *Trueperella pyogenes* nor *E. coli* were associated with SE. Results of the cited studies suggest that common pathogens associated with metritis and clinical endometritis do not have a significant role in the SE pathogenesis. It has been suggested that SE may be a response to unspecific uterine infections [19] or a prolonged inflammatory process that persists after bacterial elimination.

In several studies, cows with clinical and subclinical endometritis were shown to have increased endometrial mRNA expression and elevated serum concentrations of pro-inflammatory mediators as compared with healthy cows [20–24]. Situations of prolonged inflammation after elimination of bacterial contamination may occur when an exacerbated production of eicosanoids concurs with a low production of anti-inflammatory substances, originating a

delayed restoration of homeostasis in the affected tissues [25]. It has also been suggested that an unbalanced production of pro-inflammatory/anti-inflammatory cytokines during the first week postpartum could play a determinant role in the subsequent development of SE. A high ratio of pro-inflammatory/anti-inflammatory cytokines during the first week postpartum could lead to an excessive inflammatory response [26], whereas a low ratio of pro-inflammatory/anti-inflammatory cytokines might impair activation of inflammation and clearance of bacteria and lead to development of endometritis [22, 27].

On the other hand, diet fat levels and the type of fatty acids present in diet may affect cellular immune function [28]. Linoleic acid-enriched diets fed to dairy cows during the transition period [29] induced a pro-inflammatory status during the first week postpartum. Several studies have demonstrated that excess of adipose tissue and high serum concentrations of non-esterified fatty acids constitute risk factors for postpartum pro-inflammatory diseases in dairy cows, such as metritis or mastitis [30–32]. Innate immune response is activated when an aggressor agent is recognized by toll-like receptors (TLR). Different aggressor agents are recognized by specific TLR, which might also be activated by certain molecules in the absence of aggressor agents. For instance, lipopolysaccharides present in the cell wall of gram-negative bacteria are recognized by TLR4, which may also be activated by some fatty acids (lauric, palmitic, and oleic) [33]. Thus, an inflammatory response might be induced without the existence of infection.

In addition, the oxidative stress may contribute to an abnormal inflammatory response during postpartum [33, 34]. Increase of oxygen metabolism during postpartum would increase ROS production rate [33, 35]. Studies carried out in bovine endothelial cells evidenced that oxidative stress increased lipid hydroperoxide formation which enhanced a pro-inflammatory phenotype of these cells [36–38].

Independently of the cause of inflammation, the inflammatory status of the endometrium may have a major impact on reproduction. A direct negative effect of SE on embryo quality and survival has already been described [39, 40], which would affect conception rates. In addition, results from various studies suggest that SE may be associated to altered patterns of prostaglandin E_2 and $F_{2\alpha}$ synthesis [41, 42] which could compromise luteal function and pregnancy.

On the other hand, certain cytokines are known to play essential roles on the physiological regulation of ovarian function [43]. Cytokines are involved in regulation of follicular growth, ovulation, luteal formation, and regression [44, 45]. Inflammatory mediators, such as cytokines released in SE, may perturb this regulatory function.

3. Diagnosis

Cows with subclinical endometritis, by definition, do not show any clinical sign of endometritis, and therefore, the diagnosis of this condition requires the use of endometrial cytology, biopsy, or any other method able to evidence the presence of endometrial inflammation.

Ultrasonography has been used as a method to diagnose SE based on the presence of intrauterine fluid and on the evaluation of uterine diameter. A small amount of fluid in the uterine lumen and/or thickened uterine walls can be considered signs of endometrial inflammation. However, in various studies ultrasound was found to be less sensitive than endometrial cytology [6, 46, 47] for SE diagnosis. Presence of intrauterine fluid and a thick uterine mucosa may be normal findings in physiological situations such as estrus or early postpartum [48], and perhaps the evaluation of fluid characteristics could improve the sensitivity of ultrasound diagnosis [49]. Mariño et al. [9] found a significant relationship between presence of abnormal intrauterine fluid and SE diagnosed by biopsy but not by cytology.

Doppler ultrasonography might be useful for the diagnosis of endometritis in cattle, but it is still an unexplored tool. Debertolis et al. [50] found significantly increased blood flow in uterine arteries of cows to which acute endometritis had been experimentally induced. Whether patterns of vascular flow may differ between healthy uterus and those with SE still has to be investigated.

Endometrial cytology is considered the most reliable method for the diagnosis of SE [46], and therefore, it is the one most frequently used. Samples for cytology can be obtained by two main techniques, cytobrush and uterine lavage.

The cytobrush technique consists of connecting a cytobrush to the plunger of an insemination catheter [6] and, protected by the catheter, introducing it into the uterus as for doing artificial insemination. Inside the uterus, the cytobrush is pushed out of the catheter, gently rotated against the uterine wall, guarded back inside the catheter, and removed from the uterus. The brush is rolled onto a microscopic slide and stained. Cytology samples can be obtained from the uterine body or from one of the uterine horns. Mariño et al. [9] compared cytology and biopsy findings between the two horns of 100 bovine uteri collected postmortem and observed that samples collected from the left horn were more representative of both uterine horns than those collected from the right one.

The uterine lavage technique consists of infusing sterile saline solution into the uterus with a catheter, gently massaging the uterus to allow fluid distribution within the lumen, and recovering some of the fluid by aspiration using the same catheter. The collected fluid is centrifuged, the supernatant discarded, and the sediment smeared onto a microscopic slide. Regardless of the collection technique, cytological smears are fixed and stained using conventional stains (e.g., Diff-Quick).

Kasimanickam et al. [51] did a comparative study of the two sampling techniques and concluded that cytobrush had some advantages over uterine lavage: it was less time-consuming, was easier to perform independently of the uterine size, did not produce endometrial irritation, and induced lower degree of cell structure distortion and lower presence of erythrocytes. One disadvantage of cytobrush is that the sample is collected from a specific area of the endometrium, whereas uterine lavage provides cells from the whole endometrial surface.

Recently, Pascottini [52] described a new method for sample collection that consisted of using a paper tape rolled around the top of an insemination catheter. With this method the author

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observed less contamination with erythrocytes and a better preserved structure of epithelial cells than when using cytobrush. Moreover, this system would allow taking a sample for cytology at the same time of doing insemination.

Concerning the threshold used in different studies for the diagnosis of SE, the cutoff PMN% reported by the different authors has varied between 4 and 25% (**Table 1**) depending on the postpartum period at which the diagnosis was done.

Sampling method	**Postpartum diagnosis period**			
Cytobrush	Week 3–5	Week 5–7	≥7 week	PMN%
Lopdell et al. [53]	35.0%			>18.0%
Kasimanickam et al. [6]	35.1%			>18.0%
Heidarpour et al. [54]	13.5%			>18.0%
Kaufmann et al. [55]	12.4%			>18.0%
Barrio et al. [56]	17.6%			>18.0%
Madoz et al. [57]	21.5%			>8.0%
Dubuc et al. [58]	19.3%			>6.0%
Plöntzke et al. [59]	38.0%			>5.0%
Lopdell et al. [53]		7.0%		>18.0%
Kasimanickam et al. [6]		34.0%		>10.0%
Barlund et al. [46]		11.8%		>8.0%
Madoz et al. [57]		16.0%		>6.0%
Plöntzke et al. [59]		19.0%		>5.0%
Barrio et al. [60]		14.9%		>5.0%
Madoz et al. [57]			16.0%	>4.0%
Dubuc et al. [58]			11.1%	>4.0%
Uterine lavage				
Hammon et al. [61]	51.8%			>25.0%
Barlund et al. [46]	15.8%			>8.0%
Gilbert et al. [62]		53.0%		>5.0%
Cheong et al. [63]		25.9%		>10.0%
Cytotape				
Pascottini [52] (at AI, cows)			27.8%	≥1%
Pascottini [52] (at AI, heifers)			7.86%	≥1%

Table 1. Reported prevalence of subclinical endometritis in some studies that used different sampling methods, postpartum diagnosis periods, and PMN% cutoff values.

Uterine contamination at parturition or in the following days is unavoidable and normal, with 80–100% of animals having bacteria in the uterine lumen in the first 2 weeks postpartum [17]. Uterine contamination elicits neutrophil migration from peripheral blood to the uterine lumen and the subsequent phagocytosis of contaminating organisms by neutrophils. Prunner et al. [18] observed that uterine bacterial growth density increased from calving to 15 days postpartum and decreased from day 21 onwards and the PMN% in cytological samples decreased from calving to day 9, then increased around days 15–21, and decreased thereafter, but at each sampling period, the proportion of PMN strongly depended on bacterial counts.

Kasimanickam et al. [6] used ROC analysis to identify the PMN% above which fertility was significantly reduced, and this percentage was 18% for samples taken 20–33 days postpartum and 10% for those taken 34–47 days postpartum. Other authors also established the cutoff PMN% for diagnosing SE based on detrimental effects on subsequent reproductive performance [46, 57, 58, 64], and some [59, 61, 62] used arbitrary values. In general, most authors used PMN% thresholds of 15–18% for SE diagnosis at 21–30 days postpartum and values of 4–10% for diagnosis at later periods. Prunner et al. [18] categorized clinically normal cows at 21 days postpartum as having SE when PMN% ≥5% and found that SE-positive cytological samples had an average of 30% PMN; however, on day 28, cows previously categorized as having healthy uteri (i.e., <5% PMN on day 21) had a similar PMN% as those categorized as having SE, and for both groups, it averaged 15%. During the first month postpartum, healthy cows may show relatively high percentages of PMN in cytological samples, and therefore, diagnosis of SE during this period will be less accurate than a later diagnosis.

It has been suggested that the stage of the estrous cycle might have an effect on the proportion of PMN present in the cytology and, therefore, on the diagnosis of SE. During the follicular phase of the estrous cycle, there is an increased infiltration of PMN in the endometrium elicited under estrogenic influence [65]. Several studies [9, 52, 57] have found that the PMN% in cytological samples taken with cytobrush was not affected by the stage of the estrous cycle. However, when the SE diagnosis was done by biopsy, a higher degree of inflammatory infiltration could be observed in the follicular phase of the cycle [9]. This was because cytology only detects PMN infiltration in the superficial epithelium, whereas biopsy allows identifying inflammatory cells in deeper layers of the endometrium.

Another possibility to evidence the existence of endometrial inflammation is the use of urinary test stripes, which detect the presence of leukocytes in urine [66–68]. The diagnosis of endometritis can be done using uterine lavage fluids, or an endometrial cytobrush can be immersed in saline solution during 30 sec and then the strip introduced in the solution for 2 sec. It is a qualitative colorimetric test that showed a variable correlation with cytology. Santos et al. [66] reported a sensitivity of 96% and specificity of 98%. However, Cheong et al. [67] observed 77% sensitivity and 52% specificity. This test is rarely used in commercial dairy farms probably because it is not specifically designed for the diagnosis of endometritis.

Uterine biopsy is commonly used in human medicine as it is considered the gold standard for evaluating the human endometrium [69]. In domestic animals, uterine biopsy has been used since the 1960s to investigate causes of infertility in mares [70], and it is a routine diagnosis

method today [71]. However, in dairy cattle uterine biopsy is rarely performed by practitioners, and it is almost exclusively used for research purposes. The limited use of biopsy in clinical practice may be related with inconveniences associated to sampling time, requirement for laboratory skills, laboratory costs, and time to report [71] and also to the risk of inducing endometritis and the subsequent negative effects on fertility [72].

There are few studies using uterine biopsy for the diagnosis of SE in dairy cattle [73], and when biopsy and cytology findings were compared, the two diagnosis methods showed poor agreement [8, 9, 47]. The histopathological examination of biopsy samples gives detailed information about the degree of inflammation, distribution of the inflammatory infiltrate, or the lesions that may exist, whereas cytology only assesses the superficial layer of the endometrium [47]. Thus, it is not surprising that direct comparison between biopsy and cytology results showed low agreement. Evaluation criteria for SE diagnosis on biopsy samples, as for cytology, should be established based on detrimental effect on subsequent reproductive performance rather than on the presence of an arbitrary number of inflammatory cells [47].

4. Prevalence

The reported prevalence of SE in postpartum dairy cows has varied between 7 and 53% (**Table 1**). Such disparity among studies in SE prevalence may be due to differences in (i) postpartum period in which the diagnosis was made, (ii) PMN% established as threshold above which an endometrial cytology was considered positive for SE, and (iii) the method used to take the cytological sample, i.e., cytobrush, uterine flushing, or cytotape. In general, SE prevalence tended to be higher when the sample was collected by uterine lavage, the diagnosis was made before 30 days postpartum, and the cutoff PMN% applied was >5%.

5. Effects of SE on productive and reproductive performance

There are some discrepancies among authors concerning the effects of SE on reproductive performance of dairy cows. Whereas some authors [59, 74, 75] did not find significant effects on reproduction, many other studies described a variety of negative effects on fertility (**Table 2**). The disparity of results may not only be due to the different diagnosis criteria (e.g., postpartum period for SE diagnosis, threshold of PMN applied, etc.) used in the different studies but also to the numerous confounding factors that may have a negative effect on reproduction (e.g., poor heat detection, inadequate nutrition, insufficient cow comfort, old cows, poor semen quality, other diseases, etc.).

Subclinical endometritis has been related with the repeat breeder cow syndrome with controversial results. Whereas in some studies [78, 79] the prevalence of SE in repeat breeder cows was reported to be close to 50%, in another study [80] the observed prevalence was lower than 15%. The PMN% thresholds used in those studies differed from 3% [78] and 5% [80] to 10% [79].

Reference	Characteristics of the study	Reproductive impact	Affected parameters
Kasimanickam et al. [6]	n = 228; farms = 2; no cows with PVD; cytobrush; 20–33 DIM: >18% PMN; 34–47 DIM: >10%PMN	Adverse	Days open. Pregnancy rate
Gilbert et al. [62]	n = 141; farms = 5; no cows with PVD; uterine lavage; 40–60 DIM: ≈5%PMN	Adverse	Postpartum anestrus. First-service pregnancy rate. Services per conception. Days open. Pregnancy rate
Barlund et al. [46]	n = 221; farms = 8; no cows with PVD; cytobrush; 28–41 DIM: >8%PMN	Adverse	First-service pregnancy rate. Services per conception. Days open. Pregnancy rate
Dubuc et al. [58]	n = 1044; farms = 6; some cows with PVD; cytobrush; 35 ± 3 DIM: >6% PMN; 56 ± 3 DIM: >4% PMN	Adverse	Pregnancy rate
Plöntzke et al. [59]	n = 201; farms = 3; no cows with PVD; cytobrush; 18–38 DIM: >5%PMN; 32–52 DIM: >5%PMN	Without effect	Days to first service. Services per conception. Days open. Pregnancy rate
Burke et al. [76]	n = 78; farms = 1; no cows with PVD; cytobrush; 42 DIM: >6%PMN	Adverse	Postpartum anestrus
Green et al. [77]	n = 169; farms = 1; no cows with PVD; cytobrush; 21 ± 3 DIM: >18%PMN; 42 ± 3 DIM: >18%PMN	Adverse	Postpartum anestrus
McDougall et al. [64]	n = 303; farms = 1; some cows with PVD; cytobrush; 29 ± 2.4 DIM: >9%PMN; 43 ± 2.3 DIM: >7%PMN	Adverse	Postpartum anestrus. First-service pregnancy rate. Days open
Drillich et al. [39]	n = 48; farms = 1; no cows with PVD; cytobrush; IA: 0% PMN; embryo collection: 0% PMN	Adverse	Transferable embryo recovery rate

Reference	Characteristics of the study	Reproductive impact	Affected parameters
Fernandez-Sanchez et al. [40]	n = 41; farms = 1; no cows with PVD; cytobrush; no PMN% cutoff; donor cows in embryo transfer programs	Adverse	Transferable embryo recovery rate
Prunner et al. [74]	n = 383; farms = 10; no cows with PVD; cytobrush; 20–30 DIM: >5%PMN	Without effect	Days to first service. Services per conception. Days open. Pregnancy rate. Culling rate
Barrio et al. [56]	n = 467; farms = 1; no cows with PVD; cytobrush; 30 ± 2 DIM: >18%PMN	Adverse	First-service pregnancy rate
Barrio et al. [60]	n = 65; farms = 25; no cows with PVD; cytobrush; 30–45 DIM: >5%PMN	Adverse	Days open
Gobikrushanth et al. [75]	n = 126; farms = 1; no cows with PVD; cytobrush; 25 ± 1 DIM: >8%PMN	Without effect	Follicular development and ovulation. First-service pregnancy rate. Cows pregnant at 150 and 250 days postpartum

n, number of animals; PVD, purulent vaginal discharge; DIM, days in milk.

Table 2. Reported effects of subclinical endometritis on reproduction.

In addition to the potential effects on reproduction, endometritis may negatively affect milk production [81]. Clinical and subclinical endometritis have been related with a decrease in milk production of 0.6–1.03 kg/cow/day, reduction of milk fat and protein, and with increased somatic cell counts in milk [64, 76, 82]. Nevertheless, some authors [83] question these effects.

6. Treatment of subclinical endometritis

Antibiotics and prostaglandins $F_{2\alpha}$, combined or individually, have constituted the most common treatment for clinical endometritis postpartum. Haimerl et al. [84] and Lefebvre and Stock [85] did a critical evaluation of the scientific literature that in the last 20 years reported the use of $PGF_{2\alpha}$ alone or combined with antibiotics for the treatment of clinical endometritis in postpartum dairy cows. Both groups of researchers concluded that there was not enough clinical evidence that using $PGF_{2\alpha}$ in endometritis postpartum had a beneficial effect. And the only antibiotic that seemed to be effective for clinical endometritis was cephapirin (a first-generation cephalosporin).

In the case of SE, the treatment with $PGF_{2\alpha}$ and/or antibiotics was tested only in a few studies that cannot be easily compared as included different hormonal protocols for synchronization of estrus or ovulation, animals in different postpartum periods, and different diagnosis criteria for SE. In the studies of Kasimanickam et al. [51], Galvão et al. [86], and Denis-Robichaud and Dubuc [87], intrauterine infusion of cephalosporins was tested as treatment of SE, and Kasimanickam et al. [51], Galvão et al. [88], and Lima et al. [89] tested the use of prostaglandins. Kasimanickam et al. [51] and Denis-Robichaud and Dubuc [87] obtained higher pregnancy rates at first insemination in cows treated with intrauterine cephapirin than in control cows, whereas Galvão et al. [86] did not observe any positive effect on reproduction when cows diagnosed with SE were treated with intrauterine ceftiofur infusion. Concerning the use of $PGF_{2\alpha}$ in cows with SE, Kasimanickam et al. [51] and Galvão et al. [88] observed positive effects on reproductive performance, whereas Lima et al. [89] did not find any effect. It should be pointed out that the magnitude of the positive effects observed in some of the cited studies was dependent on other factors such as existence of ovarian activity at the time of treatment [87] or body condition [88]. The scarce number of studies done so far and the different results obtained do not allow us to draw a conclusion about the efficacy of using $PGF_{2\alpha}$ and/or cephalosporins for the treatment of SE.

Because in many cases of SE there is no uterine content or positive bacterial culture, treatment with antibiotics or prostaglandins should be expected to be unsuccessful. However, there is an inflammatory response that very likely is the cause of the negative effects of SE, and therefore, the use of nonsteroidal anti-inflammatory drugs (NSAID) would be fully warranted. Priest [5] tested the use of the NSAID carprofen, three doses administered at 3-day intervals between 21 and 31 days postpartum, in cows diagnosed with SE when the cytology showed >14% PMN at 14 days postpartum. The treatment did not reduce the incidence of SE at day 42, but increased pregnancy rate as compared with untreated control cows. However, in a subsequent study [90], cows were treated with carprofen at 1 or at 3 weeks after calving, and the treatment did not improve milk production, indicators of health or reproductive performance.

Uterine lavage with sterile saline solution is a common treatment for endometrial inflammation in mares. Uterine lavage favors the elimination of inflammatory products, such as nonfunctional PMN, and induces uterine contractions that facilitate the evacuation of any content. In addition, elimination of nonfunctional PMN favors migration of new functional PMN that is able to counter the infection [91]. In bovine, potential usefulness of uterine lavage has been described in connection with treatment of repeat breeder cows, either as the only treatment or combined with prostaglandins and/or antibiotics, assuming that many repeat breeder cows may suffer chronic endometritis [92]. In cases of SE postpartum, uterine lavage with physiological saline at day 30 postpartum was associated with a reduction of the PMN% in cytological samples obtained at day 40, but its effect on reproduction was not evaluated [93].

Other protocols that have been used for the treatment of metritis or clinical endometritis, such as intrauterine infusion of dextrose [94], ozone [95], or N-acetylcysteine combined with amoxicillin and clavulanic [96], have not been tested in cows with subclinical endometritis. Nevertheless, the effect of those substances is mainly antibacterial or mucolytic, whereas in SE there is no mucopurulent secretion and, in most cases, no pathogen bacteria.

7. Conclusions

Subclinical endometritis is a uterine inflammation probably originated by the alteration of the inflammation regulatory mechanisms. The inflammatory status may abnormally persist after elimination of postpartum bacterial contamination, which may be associated with an unbalanced production of anti- and pro-inflammatory factors. Prevalence of subclinical endometritis in dairy farms may reflect the immune status of cows, which in turn would be indicative of the metabolic status of cows in transition and, eventually, of the nutritional management of farms.

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Chapter 6

Apoptosis as the Major Cause of Embryonic Mortality in Cattle

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Additional information is available at the end of the chapter

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Abstract

Besides several mechanisms such as autophagy, necroptosis, and pyroptosis, programmed cell death (PCD) also includes apoptosis which is characterized by membrane blebbing, chromatin condensation, and DNA fragmentation which involves a number of membrane receptors and a cascade of signal transduction steps resulting in the activation of an ample number of proteases known as caspases. Even though this mechanism plays a significant role in the progressions of gamete maturation and embryo development, contributing to the formation of different organs and structures, they also lead to the death of blastomeres and, consequently, the occurrence of structural abnormalities, increasing embryo and fetal mortality. Therefore, understanding the mechanisms involved in apoptosis dysregulation may contribute to the development of new therapeutic methods to prevent various developmental abnormalities. The purpose of this chapter is to review recent research into the mechanisms of programmed cell death, focusing on apoptosis during embryo development leading to embryo mortality. The final section includes a discussion of the implication of the findings for future research on reducing embryo mortality in the bovine.

Keywords: apoptosis, bovine, embryo mortality

1. Introduction

During apoptosis, cell suicide is controlled by the genes involved in the induction or prevention of apoptosis. These genes play crucial roles in the activation/inactivation mechanisms of apoptosis, upstream/downstream of effector molecules, and in the signaling pathways, which will be reviewed below.

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In fact, apoptosis was primarily observed by Karl Vogt in Neuchâtel, Switzerland, in the year of 1842, although he did not use this term. He noticed, in the midwife toad (*Alytes obstetricans*) embryos, that cells in the notochord, a cartilaginous skeletal structure, would disappear during development, being replaced by cells of the vertebrae. Despite having documented that some cells disappeared during development, Vogt did not focus his research on this phenomenon. This problem was addressed again only when Walther Flemming, using more advanced staining techniques on the cell nucleus, observed what he called chromatolysis, the diminishing of nuclear material in dying cells. He provided then a more precise description of the whole process in 1885. However, only in 1965, this topic was addressed once again [1].

Although PCD is a term often used as a synonym to the designation of apoptosis, which indicates an endogenous cell suicide program used to eliminate useless or damaged cells, in fact, other forms of regulated cell death, e.g., the autophagy, necroptosis, and pyroptosis, included in the non-apoptotic pathways of PCD exist [1–3]. During embryogenesis, the elimination of the cell by apoptosis is an important way of molding the tissues and shaping the body. But apoptosis occurs not only during embryo development. It occurs also after birth and during cell turnover and tissue homeostasis [4]. For example, it occurs in brain cells, which undergo apoptosis either prior to or after birth, to eliminate excess brain cells and streamline nerve impulses. Apoptosis also occurs in some cancers, where activation of the apoptotic pathways may prevent the spread of neoplastic cells and contain the cancer. However, dysregulation of apoptosis is also associated with aging decline, multiple diseases (e.g., Alzheimer's disease and amyotrophic lateral sclerosis), and malignant cancer, as well as abnormalities in development [5–7].

Embryo mortality is generally defined as the loss of the conceptus before day 42 of pregnancy, i.e., before the complete development of the organs. It is well documented that about 30% of all embryos are not able to survive. Of all lost embryos, approximately 80% of the embryos are lost before day 17, while 10–15% are lost between days 17 and 42. Only 5% of pregnancies are lost after the 42nd day [8].

Although diverse causes for early mortality exist, it is well documented that short-term exposure to heat stress before and after insemination results in low conception rates or embryonic death, due to an elevated uterine temperature, affecting cattle reproductive performance mainly in spring and summer. Other than the temperature, nutritional factors, infectious agents, and animal's welfare in general, contribute to low conception rates resulting from embryo mortality. Whether this is due to an abnormal hormonal environment, such as reduced progesterone secretion, or the ovulation of a defective oocyte has not been determined [8].

Previous *in vitro* and *in vivo* studies by our group [2] demonstrated that situations causing discomfort to cattle were associated to the activation of the genes responsible for embryo apoptosis, including Cx43, CDH1, DNMT1, and HSPA14, resulting in an acceleration of apoptosis in embryonic cells.

The apoptosis mechanism acts on three levels [9]: at the membrane level, where specific receptors mediating death signals have been identified; at the nuclear level, as the genome itself contains genes that are transcribed in response to molecules triggering the apoptotic process

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(e.g. p53); and at the cytoplasm level, where the signal transduction pathways actuate in response to diverse stimuli. These signal transduction pathways are different for each initial receptor and generally integrate cysteine proteases—the so-called "caspases" (CASP)—which are the central regulators of apoptosis. The initiator caspases (including, CASP-2, -8, -9, and -10) are closely coupled to pro-apoptotic signals. Once these caspases are activated, they cleave and activate downstream effector caspases (including CASP-3, -6, and -7), which in turn execute apoptosis by cleaving cellular proteins.

2. Mechanisms of apoptosis

Apoptosis has been described as a stepwise process [3], which includes cell's shrinking and loss of intercellular connections, condensation of chromatin, blebs appearing in the membrane cells, collapse of nucleus, and finally the collapse of the cells in different fragments—the so-called apoptotic bodies—further engulfed by the phagocytes or the neighboring cells [9].

The first step respects the onset of apoptosis, which can be achieved by different pathways: the intrinsic pathway (also named mitochondrial pathway) and the extrinsic pathway (also called dead receptor pathway) are the two best understood pathways. But some authors [9–11] refer to an additional pathway involving T-cell-mediated cytotoxicity and the perforin-granzyme-dependent killing of the cell via either granzyme A or granzyme B. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution, pathway, which is initiated by the cleavage of CASP-3 [9] (**Figure 1**), starting step two of the process. Apoptosis is considered an irreversible process from this point on; once the effector caspases are activated, the death of the cell always occurs [12].

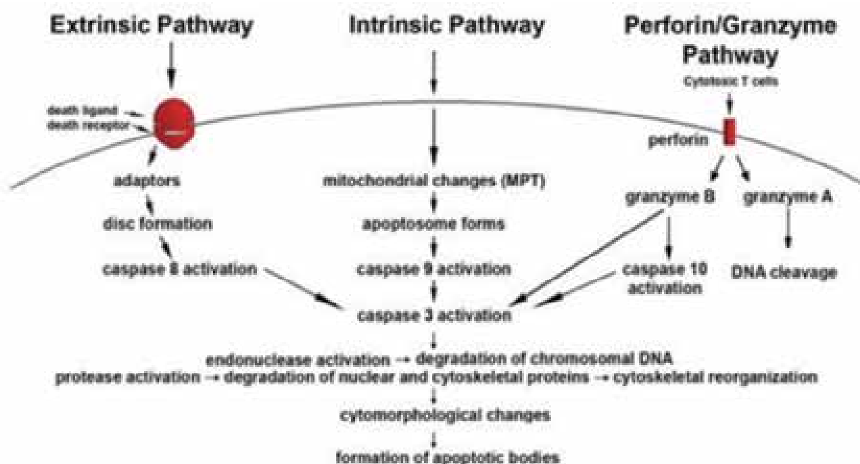

Figure 1. Schematic representation of apoptotic events (adapted from [9]).

Resulting from the action of activated effector caspases, nuclear chromatin marginates and condenses, while the DNA undergoes fragmentation. Taking a morphological approach, the chromatin condensation that occurs in an interphase nucleus upon incubation in a mitotic extract is very similar to chromatin condensation occurring in an apoptotic nucleus as DNA ends up being broken up into smaller pieces by enzymes that are activated as a part of the apoptotic program [13]. This fragmentation can be visualized by gel electrophoresis since it produces such a characteristic pattern. Terminal deoxynucleotidyl transferase dUTP nick-end labeling is a commonly used assay that can detect DNA fragmentation. This method is usually referred to as TUNEL assay. This assay labels 3′-OH DNA ends that are produced during apoptotic DNA cleavage. The characteristic nuclear feature of apoptosis can also be histologically seen as shrunken or fragmented nuclei when the cells are labeled with DNA-binding dyes, such as the Hoechst [14].

Nowadays, there are several fluorescent probes that can be used to monitor caspase activity in living cells.

After DNA fragmentation, the cytoplasmic membrane bulges irregularly, due to the decoupling of the cytoskeleton from the plasma membrane. The bulge sooner or later will bleb off from the plasma membrane, taking part of the cytoplasm with it. The irregularly shaped cell membrane loses the ability to maintain the intercellular connections with the neighboring cells, and also the integrity required to maintain transmembrane gradients [15]. The blebbing of the apoptotic cellular membrane can be easily observed on electron micrographs. It is usually described as an early feature distinguishing apoptotic and necrotic cell. Membrane blebbing has been associated to caspase-mediated activation of the Rho-associated Kinase ROCK I [16]. The membrane blebbing is followed by cell fragmentation into membrane-bound apoptotic bodies (**Figure 2**), containing cytoplasm and highly packed organelles. The recruitment of local phagocytes ensures the clearance of dying cellular debris, which is why apoptosis does not incite an inflammatory reaction [40]. Besides, they also have a protective role for surrounding cells and may serve as a signal for the recruitment of progenitor cells, for tissue regeneration. Recently, it has also been hypothesized that some microblebs could be released from the apoptotic cell that could be used as a biomarker for disease [17].

Externalization of phosphatidylserine residues at the outer plasma membrane of apoptotic cells may be detected via Annexin V, a dye that signals the existence of disrupted cell membranes [19]. Bounding with FITC-labeled Annexin V (for fluorescence) allows the identification of apoptotic cells in fluorescent microscopy. A strength in this technique is the sensitivity (they can detect a single apoptotic cell at an early stage of the process). However, it has the disadvantage to also label the membranes of necrotic cells, since they also present loss of membrane integrity. Annexin is often used in conjunction with a vital stain, such as propidium iodide, to distinguish between cells in early or late apoptosis, or the propidium iodine and trypan blue to differentiate cells in apoptosis and necrosis [9].

2.1. The extrinsic pathway

The extrinsic pathway is activated by extracellular ligands that bind to the cell-surface death receptors (e.g., FAS ligand or other TNF superfamily receptors), a transmembrane protein, which leads to the activation of inducer caspases 8 and 10 [20]. Different ligands have their specific cell death-receptors, and thereby, different stimuli may use different receptors.

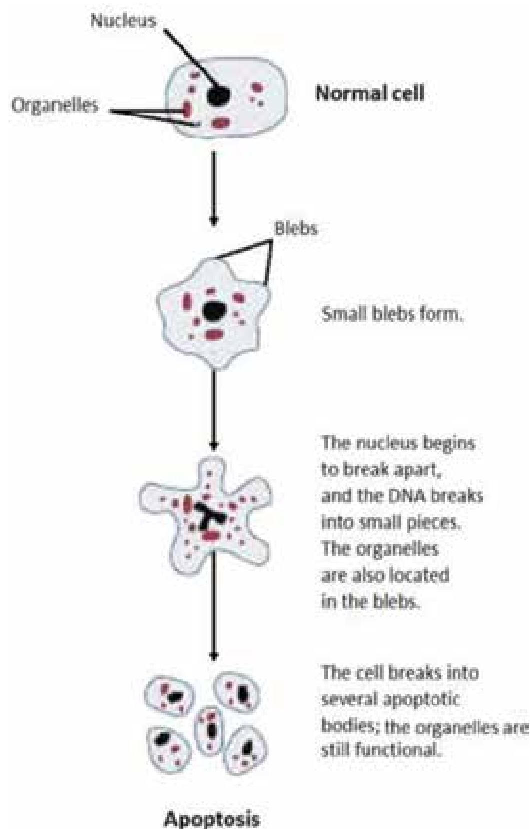

Figure 2. Schematic representation of apoptosis (adapted from Wikipedia [18]).

Activation of the cell death receptors at the cell surface induces the formation of a death-inducing signaling complex (DISC) that activates CASP-8, which is released into the cytosol to cleave the effector CASP-3, -6 and -7 (**Figure 3**). These caspases are associated with the breakdown of the cell cytoskeleton and the activation of an endonuclease, which will induce the DNA fragmentation [9].

The intrinsic pathway can also link to the extrinsic pathway, as a way to amplify the apoptotic phenomenon. This link is established through the interaction of CASP-8 with one of the pro-apoptotic molecules of the Bcl-2 family, that will stimulate the intrinsic cascade. Besides the DISC activation of CASP-8, this caspase can also be activated by a feedback loop derived from the intrinsic apoptotic pathway mediated by the release of cytochrome C from the cell mitochondria [21].

2.2. The intrinsic pathway

The intrinsic pathway is activated by intracellular endogenous or exogenous stimuli, such as ischemia, oxidative stress, or in response to chemical stressors or other stimuli originating chromatin fragmentation. Disruption of the intracellular homeostasis is at the origin of the release of signals for cell suicide, like the release of cathepsins from the lysosomal lumen [22].

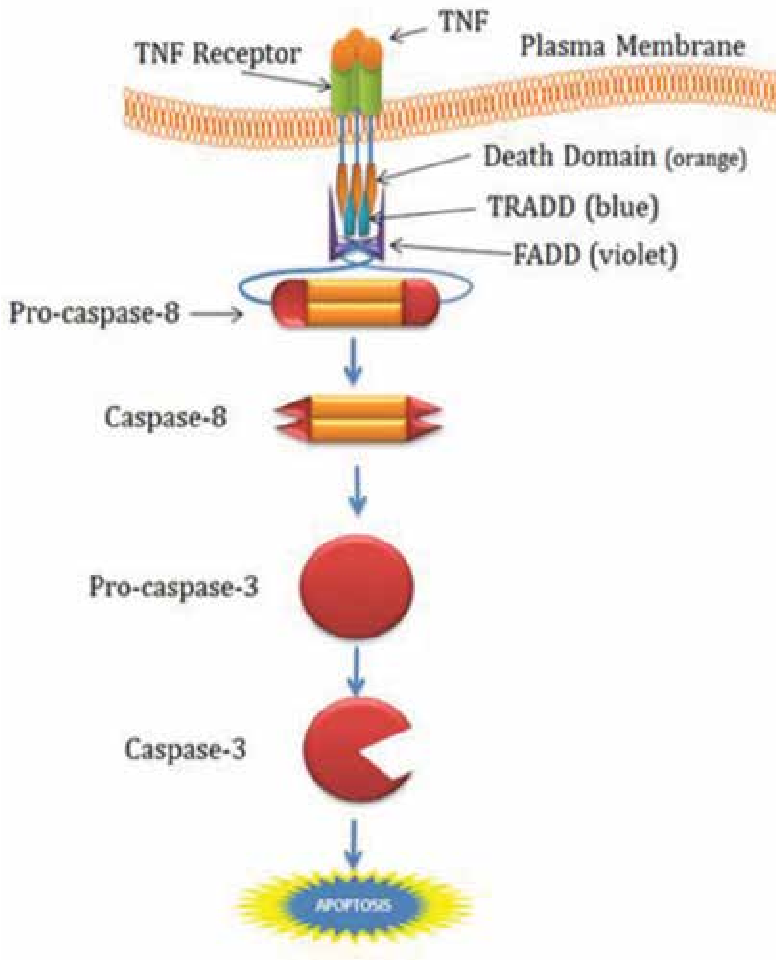

Figure 3. Extrinsic pathway of apoptosis (adapted from [9]).

The mitochondria are key players in this pathway [23], although the endoplasmic reticulum has also been implicated. After an apoptotic insult, the permeability of the mitochondrial membranes is disrupted, allowing the influx of ions and the efflux of some molecules localized within the mitochondria (e.g., cytochrome C) and the activation of Bax/Bak channels and APAF-1 [22]. These events lead to the activation of CASP-9, which in turn cleaves and activates the executioner CASP-3, -6, and -7 [24]. This pathway represents the caspase-dependent cascade, but the intrinsic apoptotic pathway also presents a caspase-independent cascade of events [23].

Although the caspase activation may originate from the death of the cell, in mammals, in particular circumstances, cells can undergo caspase-independent apoptosis. This form of apoptosis is mediated by the mitochondrial dysfunction, with loss of the mitochondrial membrane potential, which originates the translocation of an apoptosis-inducing factor (AIF) and a mitochondrial-derived protease—the endonuclease G—into the nucleus. In there, they

induce large-scale DNA fragmentation with subsequent peripheral condensation of chromatin that results in cell death [25]. Although the caspase-dependent and caspase-independent apoptotic pathways are separate, there is evidence of crosstalk between the two [26].

In the extrinsic pathway, the cell death-inducing signal for the programmed cell death is triggered by an external stimulus. For receiving such an external death-inducing signal, cells possess plasma membrane receptors specific to each stimulus and thus the extrinsic signaling of apoptosis, which in most cases is a cytokine, is also known as the receptor-mediated programmed cell death pathway. The most studied cytokine to induce extrinsic pathway of apoptosis is an extracellular messenger protein called tumor necrosis factor (TNF), produced by the cells of the immune system in response toward adverse conditions (**Figure 3**).

3. Apoptosis in female gametogenesis

In females, oogenesis is the process by which the female gametes, the oocytes or ova, are created. It begins with the allocation of the embryonic precursors of adult gametes, known as primordial germ cells (PGCs), into the gonadal ridges and forms the primordial sex cords, which will ultimately originate primordial follicles. Besides ovarian cell death that has been studied for over a century, recently in March 2018 [27], Regan and collaborators postulated that apoptosis is an integral part of normal ovarian cell's development and has limited predictive capability regarding oocyte quality or the ensuing pregnancy rate. In the ovary, the mechanisms underlying decisions of life and death involve cross dialog between pro-apoptotic and pro-survival molecules [28]. Even though apoptosis in the post-pubertal ovary is frequently observed in granulosa cells at all stages of follicle development, in fetal life, it only occurs in the oocyte [28]. In consequence, a large population of ovarian follicles in mammal ovaries is lost, limiting the number of ovulations and restricting the full reproductive potential of a species [29, 30].

Atresia of ovarian follicles has been divided into three phenotypes, each one presenting a different mechanism of initiation and regulation [27]. In the "antral atresia," the middle prolific layers of granulosa cells are affected by the apoptosis, which progresses to the antrum. The "basal atresia," occurs in the granulosa cells closest to the basal lamina, in very small antral follicles; these cells prematurely luteinize and begin to produce progesterone, but they do not complete luteinization and become apoptotic. The third form of apoptosis, often named as "terminal differentiation apoptosis," occurs in the preovulatory follicles and resembles that of the epidermal skin cells sloughing off, with the granulosa cells being shed into the antral fluid [27, 31]. In the male, apoptosis also has a significant relation with fertility.

4. Apoptosis and the gamete's quality

Fewer than 10% of oocytes collected in IVF programs become live births [32], leading to research on the relationship between apoptosis and oocyte quality. As in mice, the level of granulosa cell apoptosis increases in older IVF animals, in association to a decrease in the

oocyte quality, which is reflected in lower fertilization, pregnancy, and live birth rates [33]. In 1996, Sugino and collaborators [34] reported the relationship between the frequency of apoptotic granulosa cells and the size of the follicles in IVF programs. These findings were supported by several authors [35–37] leading to the hypothesis that the amount of cell-free DNA level in the follicular fluid samples obtained during follicle aspiration would correlate with the level of apoptosis in granulosa cells and thereby could be used as a predictor of oocyte quality and IVF-embryo transfer outcome.

5. Apoptosis after fertilization

In 2010, our team [14] studied the relationship between the arrest of bovine embryos in different stages of development and the level of apoptosis in embryonic cells, as measured by the TUNEL assay. It has been demonstrated that among embryos 7 days after IVF, those of nine cells to morula presented a higher incidence of apoptotic cell ratio (ACR) when compared with blastocysts. It was further demonstrated that embryos with higher ACR also showed higher cytoplasmic fragmentation and that higher ACR was associated with embryonic arrest. These findings lead the authors to hypothesize that an apoptosis level above a given threshold could be harmful to embryo development [14]. Notwithstanding, further research is foreseen to determine the pathways of the apoptotic process and especially to establish the ACR threshold detrimental to embryo development since nowadays it is well accepted that early cleaving embryos will result in a significantly higher proportion of good quality embryos compared with late cleavage (62.5 versus 33.4%, $P < 0.0001$) [38].

Besides, a lower apoptotic cell ratio in early cleaved embryos may anticipate higher embryo qualities. During compaction and blastulation of bovine embryos, it is often observed excluded cells between the developing embryo and the zona pellucida, which have poor gap junction communication with the embryo. Arrested and developing embryos contain different proportions of cells with the classic features of apoptosis, [39] including cytoplasmic, nuclear, and DNA fragmentation. In the embryo, the larger the number of apoptotic cells in an embryo, the lower is the ability of that embryo to survive in culture [40].

6. Apoptosis in the implantation process

Endometrial receptivity depends on a complex interplay of many factors that integrates the diverse mechanisms regulating tissue homeostasis, including apoptosis [41]. Endometrial function and in particular the endometrial receptivity are critical limiting factors for pregnancy success because, for implantation, pregnancy, and subsequent birth of the offspring, the endometrium should be ready to accept and interact with the embryo. Embryo implantation depends on some morphologic and biochemical modifications of the endometrium during the estrous cycle, orchestrated by the action of ovarian steroids on its receptors. These changes are exerted by cytokines, growth factors, adhesion molecules, transcriptional factors, and many others [42].

As it happens in other hormone-responsive cells, the endometrium presents a cyclic pattern of apoptosis, both in the epithelial and stromal cells, that allows the constant regeneration of the tissue and ensures fertility [43]. In women, it has been shown that the equilibrium between cell proliferation and apoptosis controls the endometrial cells, dictating its fate toward destruction and shedding at menstruation or toward survival, and prepare the mucosal layer of endometrium for the implantation of the embryo [44]. Endometrial receptivity represents a very short, self-limited period in which the endometrium does not reject the embryo [45].

Von Rango and collaborators [46] detected signs of apoptosis in the deep glandular epithelium and stroma at the beginning of the implantation window, in the human endometrium, that extended to other endometrial epithelia later on the luteal phase. The authors hypothesized that this pattern of apoptosis might have implications for the decidualization processes formation in endometrium during the late secretory phase. Supporting this hypothesis, Joswig and collaborators [47] demonstrated that apoptosis occurs in the uterine epithelium of the implantation chamber, as detected by the TUNEL assay and the immunolocalization of active CASP-3. It was also sporadically observed in decidual cells adjacent to the implantation chamber [47]. Apoptosis may, therefore, contribute to tissue remodeling during implantation and establishment of the placenta. Zhang et al. [48] demonstrated that the embryo controls the transcription of the apoptosis-inducing factor (AIF—a participant in the intrinsic, caspase-independent pathway) in the maternal endometrium around the time for implantation, both temporally and spatially. AIF transcription is maintained in basal levels in the surface and glandular superficial epithelia, those epithelia that interact with the trophoblast in mice. During the invasion phase, in implantation, its transcription increases in the sub-luminal stroma at the implantation sites, but not in the interplacentary sites. The authors concluded that apoptosis is vital for embryo implantation, decreasing the apoptosis in the apposition phase and contributing to the success of the blastocyst invasion. Any disturbance in the apoptosis pattern could lead to infertility and recurrent pregnancy lost.

However, most studies available were developed in species with a decidual placenta. So, it is possible that species-specific differences exist related to the physiology of implantation, such as the moment for implantation and the type of implantation, that are still to be elucidated. The detection of apoptosis in the endometrium during embryo implantation surely gives important information about the endometrial receptivity. Besides, the exact mechanisms and factors mediating the apoptotic process in the endometrium are not fully understood in many species, additional research of this problem would possibly let us to comprehend the mechanisms of endometrial receptivity better and to improve new predictors of IVF results.

Apoptosis was also detected in the trophoblast, under physiological conditions [49], which was mediated by the Bcl-2 gene and the Fas receptor [50, 51].

During placentation, ultrastructural studies showed that apoptosis contributes actively to the shaping and reorganization of fetal membranes [52], in a mechanism associated with the Fas-mediated signaling pathways. In post-implantation embryos, apoptosis is involved in organogenesis and in processes such as removing anomalous, inappropriate, nonfunctional, or damaging cells, and adjusting cell numbers. Although being a crucial phenomenon for embryo viability and pregnancy survival, this topic falls out of scope in this review.

7. Conclusions

Apoptosis is a process that tackles diverse specific intracellular signaling cascades that culminate in the enzymatic activation leading to programmed cell death. The enzymes most widely linked to apoptotic cell death are caspase enzymes. Caspase enzymes are cysteine proteases that are present in cells as zymogens until activated. Even though the mechanisms of apoptosis are present through the entire life, they start at the gametic formation as well as during all stages of embryo development. It first appears in the 32- to 64-cell embryo and can be demonstrated during the whole embryogenesis, when it plays an essential role in virtually all of the stages of development necessary to produce a normally developed newborn. It is also crucial for embryo-maternal interaction and seems to regulate the implantation and placenta formation. Evidences have accumulated that the formation of inborn anomalies or intrauterine death, induced by different developmental toxicants, result from distortions of the normal pattern of PCD in the embryo. Various chemical agents and physical factors have been shown to exert their effect by disturbing the apoptotic process occurring during gametogenesis. For that same reason, in response to environmental stressors, apoptosis may be an important event in embryo losses in early pregnancy. The mechanisms of apoptosis as well as its regulation in the early embryonic period has been studied in women or in rodent species, as models for human fertility, but limited information exists regarding farm animal species. This dearth of evidence opens new avenues for research on the apoptosis role in early pregnancy and fertility of livestock.

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Conflict of interest

The authors declare, for all legal purposes, the absence of any conflict of interest related to this paper.

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Chapter 7

Embryo Manipulation Techniques in the Rabbit

María-Luz García

Additional information is available at the end of the chapter

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Abstract

Rabbits are both productive and classic laboratory animals. Some particularities of female reproductive physiology make the rabbit an extraordinary model for the study of embryology and assisted reproductive techniques. For instance, as the ovulation is induced, the embryo development can be known with accuracy. Embryos are surrounded by a mucin coat which is crucial to prevent embryo mortality. Besides, the anatomy of the uterus does not allow embryo transmigration between both uterine horns, and so it is possible to test different reproductive techniques. Knowledge on early embryo development, and on influencing factors, has allowed to develop new insights into embryo manipulation, such as recovery, transfer, cryopreservation, *in vitro* fertilisation, cloning, or transgenesis. Also the rabbit may be used as a model for human reproductive health, because rabbit embryo and feto-placental development are similar to the human. This chapter reviews the aspects of the reproductive physiology in the female rabbit and discusses some embryo manipulation techniques available in the species.

Keywords: development, embryo, rabbit, reproductive techniques, survival

1. Introduction

Rabbit (*Oryctolagus cuniculus*) is a livestock species reared either for the production of hair, skin or meat [1] or as an experimental reference for other species, such as pigs or humans [2]. In rabbit meat production, litter size is an important economic trait [3, 4], and has been the objective in selection programs [5]. Litter size components are ovulation rate, fertilisation rate and prenatal survival, the latter component being defined by the embryo and foetal survival. Prenatal survival is around 30% [6], 15% corresponding to embryonic period. Therefore, knowledge of rabbit embryology is crucial for rabbit production.

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Research in embryo biology using the rabbit as a model is associated with a number of well-known pioneer investigations on reproductive biology, e.g., fertilisation [7], oviduct physiology [8], embryo survival [9], prenatal mortality [10], experimental embryology [11], embryo *in vitro* culture [12], preimplantation embryo development *in vivo* and *in vitro* [13, 14] and embryo cryopreservation [15]. The reproductive characteristics of the rabbit have led to the development of embryo technologies with applications in genetic improvement, in the spread of genetic material or the study of diseases.

2. Reproductive particularities of the female

Female rabbit has certain physiological and anatomical characteristics, which make it especially important for the study of embryology and the application of embryo reproductive techniques.

The rabbit belongs to the few species in which ovulation is induced by mating [16, 17]. The coitus leads to the nervous stimulation of the vagina, triggering the production of gonadotropin-releasing hormone (GnRH) in the hypothalamus. Under the influence of GnRH, the anterior pituitary secretes the gonadotrophins and the follicle-stimulating hormone (FSH), which regulates follicular development, and the luteinizing hormone (LH), which leads to ovulation [16, 17]. In the absence of a mating stimulus, ovulation can be triggered by hormonal treatment, with either GnRH analogues [18–20] or human chorionic gonadotrophin hormone (hCG) [21]. As a consequence of induced ovulation, the age of the embryos [hours post coitum (hpc)] is precisely known.

Rabbit has a short reproductive cycle. Female sexual maturity occurs at about 17–20 weeks of age, depending on lines [5]. Gestation lasts for 31 days. Female shows oestrus early in post-partum, during lactation. Different reproductive systems can be used in rabbits' production, according to the production system: intensive (mating 4 days post-partum), semi-intensive (mating 11 days post-partum), semi-extensive (mating 18 days post-partum) or extensive (mating after weaning) [22]. Weaning takes place at 28 days of lactation. This implies that females sustain lactation and gestation simultaneously, except for the extensive system, but this overlap depresses sexual receptivity, ovulation, fertilisation, implantation and embryo survival due to hormonal antagonism between prolactin and gonadotropins [23, 24].

The morphology of the rabbit uterus represents a uterus duplex, i.e., constituted by two separated fully functional uterine horns and cervices opening into a sole vagina [2]. This morphology allows the transfer of two sets of embryos into the same recipient female without the occurrence of inter-horn migration. So, this species is particularly suitable to study the prenatal survival [25].

3. Reproductive cycle, ovulation and fertilisation

Female rabbits do not have a typical oestrus cycle [26]. Their period of sexual receptivity is influenced by the development of follicular populations. The receptivity can be identified by the colour red or purple of the vulva [27]. The development of follicles occurs normally in waves, with 5–10 follicles in each ovary with an oocyte inside each. The follicles produce

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oestrogen for 12–14 days. After this period, if there is no ovulation, the follicles degenerate, the oocytes are reabsorbed, and the oestrogen and sexual receptivity decrease. After about 4 days, a new wave of follicular growth begins. So, the reproductive cycle lasts 16–18 days, of which 12–14 days, the female is receptive and the remaining 4 days, it does not accept the male.

If ovulation takes place, the follicles become corpus luteum, starting a luteal phase characterised by high levels of progesterone that block new follicular waves. Whenever fertilisation, implantation and gestation occur, the corpus luteum is maintained throughout pregnancy. The oocytes are captured by the ovarian bursa of the infundibulum and are quickly transported through the ampulla, due to muscular contractions and ciliary activity. Fertilisation occurs shortly after ovulation in the ampulla by capacitated sperm that has already been present for 10–12 h in the female tract [28]. Fertilisation rate is generally high, about 95% [29, 30].

But if no fertilisation is achieved, or early embryonic losses are produced, the uterus produces prostaglandin-2α ($PGF_{2\alpha}$) 17 days after ovulation, which has luteolytic action. The progesterone levels decrease and a new wave of follicular growth is produced.

4. Embryo development

After fertilisation, the resulting zygote progresses through the next stage of development rather quickly in the isthmus and uterotubal junction. **Table 1** shows the timing of *in vivo* embryo development between 24 and 84 hpc. Overlap of different embryo developmental stages is commonly observed [30–33], and it could be related to the duration of ovulation or the oviductal and uterine fluid compositions [34, 35]. It is known that a high ovulation rate increases the overall duration of ovulation, and later ovulating follicles may be fertilised later [36].

Early embryo losses, defined from fertilisation to implantation, have been estimated at around 15% [6] and they may result of an inherited abnormal development, an asynchronism between the stage of embryo development and the uterine environment, or to an inadequate steroidogenic pattern [14, 37].

hpc	Zygote	2-cells	4-cells	8-cells	16-cells	Early morulae	Compacted morulae	Blastocyst	Expanded blastocyst
24	X	X							
28		X	X						
30		X	X	X					
48					X	X	X		
62						X	X		
72						X	X	X	
84								X	X

hpc: hours post coitum.

Table 1. Timing of *in vivo* embryo development in rabbits.

4.1. Embryo coats

The extracellular coverings of rabbit embryos are structurally more complex than in most mammalian embryos. The peculiarity of rabbit embryos is that they are surrounded not only by the zona pellucida but also by a mucin coat (**Figure 1**).

Zona pellucida is an extracellular embryonic coat that surrounds the early embryo (**Figure 1**). Zona pellucida proteins are synthesised by the oocyte and the granulosa cells to form concentric layers consisting of cross-linked zona proteins [38]. The steadiness of zona pellucida allows the intrazonal diameter of the embryo to remain constant (around 120 μm) up to 72 hpc [33, 39]. The zona pellucida thickness itself ranges between 15.9 and 19.2 μm [32, 40]. The zona pellucida selects morphologically normal spermatozoa [41], indices the acrosomal reaction [42] preventing polyspermy [43] and protects early embryo integrity during the transport through the oviduct [44]. It affects fertilisation; a thicker zona pellucida was found in oocytes with failed fertilisation collected from the oviducts compared with those of embryos [40]. Moreover, recent studies have shown that zona pellucida thickness is 8% lower in females under heat stress than in thermal comfort conditions, decreasing the number of normal embryos [45].

The mucin coat is a layer of acid mucopolysaccharides, which is deposited on the embryos during the passage through the oviduct [46]. The mucin coat thickness depends on the time spent in the oviduct [46], and it is half the thickness for embryos at 48 hpc (around 50 μm) than at 72 hpc (around 100 μm) [33]. The thickness of the mucin coat is essential for rabbit embryos to develop at term because it physically prevents the embryos from direct exposure to a deleterious uterine environment and allows them to expand until the appropriate time for implantation [46]. When *in vitro* embryos are transferred into recipients, the lack of a mucin coat predisposes to subsequent failure of pregnancy [47].

The embryo is covered by the zona pellucida and a mucin coat until 72–96 hpc. The zona pellucida disappears by then, and it is substituted by neozona (4.5 days) when the blastocysts enter into the uterus, while the mucin coat is covered by a new layer, named gloiolemma, around 6 days of gestation [38].

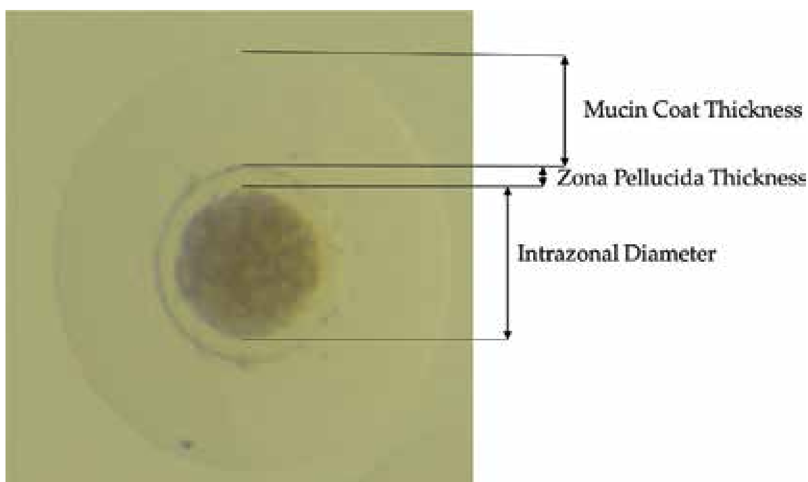

Figure 1. Rabbit compacted morulae. The image shows the measurements commonly used to assess early rabbit embryos.

5. Rabbit pregnancy

Gestation depends upon the early embryo's signalling its presence to the maternal system, a process termed maternal recognition of pregnancy [48]. The maternal recognition does not occur until the end of the first third of gestation [49], when the presence of the embryos is required to induce continued progesterone secretion by the corpus luteum.

Rabbits have a decidual and discoid haemochorial placenta [50]. The individual weight of the foetal placenta is higher than the individual weight of the maternal placenta, and both are higher for a live foetus than dead foetus [51]. Concerning the position in the uterus, the heaviest maternal and foetal placentae, and foetuses, are located near the oviduct [51, 52], probably due to the greater uterine space per foetus and higher blood flow in this region [53].

Abdominal palpation makes the simplest pregnancy diagnosis method, and it can perform by 12 dpc (days post coitum). To know not only the number of implanted embryos but also the ovulation rate, estimated from the number of corpora lutea in the ovary, it is necessary to perform a laparoscopy. Parturition takes place after 31 days of gestation.

6. Embryo cryopreservation

Since the 1970's, rabbit embryos can be cryopreserved and stored with optimal efficiency [54]. Embryo cryopreservation can be used as a tool in setting up genetic resources banks that preserve the genetic diversity and protect against loss through diseases or hazards [55, 56]. The establishment of control populations from cryopreserved embryos of genetically selected lines allows estimating the response to selection [6]. It further enables the mobility of genetic material in animal breeding, facilitating the diffusion of genetic improvement of animals with higher genetic value [57].

The first cryopreservation protocols were based on freezing. However, these have been replaced by the vitrification technique because it is an easier and cheaper technique. Vitrification permits the rapid cooling of the liquid medium avoiding ice crystal formation by the use of high levels of cryoprotectants. Several protocols have been developed for embryo vitrification, providing a survival rate *ca*. 60% after transfer [58–62]. Some vitrification procedures and solutions applied in rabbit [58, 61, 62] are similar to those used in human [63, 64]. An example of a protocol is one that is carried out in two steps, at 20°C. First, embryos are placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl sulphoxide (3.5 M) and 12.5% (v/v) ethylene glycol (4.4 M) in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.2% (w/v) of bovine serum albumin (BSA). In the second step, embryos are suspended for 1 min in a solution of 20% (v/v) dimethyl sulphoxide, and 20% (v/v) ethylene glycol in DPBS supplemented with 0.2% of BSA. Thereafter, the embryos suspended in the vitrification medium are loaded into 0.25 ml plastic straws, and two sections of DPBS are added at the beginning and the end of each straw, separated from the embryo containing medium by air bubbles. Finally, straws are sealed, identified and plunged into liquid nitrogen at −196°C [62].

Devitrification is performed by immersing the central and the final sections of the straws in a water bath at 20°C for 10 s. The vitrification medium is eliminated in two steps. First, embryos

are expelled with the vitrification medium into a solution of DPBS with 0.33 M sucrose for 5 min; then, embryos are washed in a solution of DPBS for another 5 min [62]. The purpose is the elimination of the high concentration of cryoprotectants, which are toxic for embryos, as soon as possible.

The most recommended embryonic stage for cryopreservation is the compacted morulae. However, by different methodologies, it is also possible to cryopreserve cells from oocytes to blastocysts [65–67], bisected embryos [68] or cloned embryos [69].

A complete cryopreservation procedure involves several embryo manipulations such as *in vitro* handling, the exposure to toxic concentrations of cryoprotectants, or transfer into a female reproductive tract. The success of the vitrification-thawing procedures depends on the concentration and composition of the vitrification solution, the cooling and thawing conditions, the procedure used to dilute embryos from the vitrification solution, and the volume of the vitrification solution [70]. The genotype of the embryo and the genotype of the recipient female also affect the efficiency of the vitrification-thawing procedure [71].

Retarded embryonic development is produced in cryopreserved embryos, and it may be due to a slow and gradual restoration of normal metabolic and synthetic activities of the thawed embryo [72]. So, asynchrony between vitrified embryos and recipient females is applied to obtain higher rates of embryos developed to term [73]. Cryopreservation and transfer procedures affect the RNA expression [74], and consequently the placental transcriptome and proteome [75]. Vitrification also affects foetal survival and growth and modifies the placental development at term [60, 76] and growth from birth to adult age [77]. Nonetheless, positive long-term effects of cryopreservation and transfer procedures on female offspring reproduction have been reported, namely in the litter size and the number of newborns alive at birth [78].

7. Embryo reproductive techniques

Expanding knowledge on the embryology of the rabbit, the effective cryopreservation of embryos and the reproductive particularities of the rabbit allowed the development of embryo reproductive techniques. These techniques have been successfully applied in the rabbit industry and have been extrapolated to other species. Next, reproductive technologies related to the embryo are discussed, such as the embryo recovery and transfer, *in vitro* fertilisation, cloning and production of transgenic embryos.

7.1. Embryo recovery and transfer

The ability to recover and transfer preimplantation embryos has numerous associated applications which are inevitably linked to translational molecular genetics, cell biology and assisted reproductive technology [79]. In 1890, the first successful mammalian embryo transfer was performed in rabbits [80]. Nowadays, this technique has become a routine practice in human medicine [81].

Embryo transfer includes the generation of preimplantation embryos along with the development of those embryos until term in different recipient females. Production of preimplantation

embryos can be achieved *in vivo* or *in vitro*. In both cases, superovulation protocols can be applied to ensure the maximum number of embryos recovered per donor. The classical protocols have been performed with equine chorionic gonadotropin (eCG) or FSH (**Table 2**) [21, 82–84, 86–88]. But, both the number and quality of the oocytes obtained are highly variable when eCG is administrated [21, 83, 86]. The use of LH on super stimulation has been studied using porcine FSH (pFSH). The results are also variable due to the different LH concentration present, contamination from other hormones, inconsistencies within and among batches, and the possibility of the spread of diseases [88]. Nowadays, because of break-through in recombinant technology, it is possible to easily dispose of LH and FSH in an isolated manner [81], and the treatment with recombinant human FSH (rhFSH) alone or supplemented with LH is effective in stimulating superovulation without affecting the embryo quality [85].

Fertilisation of the oocytes can be achieved by natural mating or artificial insemination, if embryos are produced *in vivo*. Artificial insemination is usually performed with fresh or cooled semen stored for short periods of time (under 36 h) [89, 90], and obtains a high fertility rate and prolificacy. However, when fresh semen is used the sperm concentration per ml should be 4 million [91], while it should be increased to 15 million if the semen is refrigerated [89]. Fresh or cooled semen is used in the rabbit industry to improve breeding management [22]. However, frozen semen presents poor fertility after thawing [92, 93] and it is used mainly for conservation of banking resources, international exports and research [94].

A good understanding of the chronology of events that follows the ovulation is crucial to the recovery of preimplantation embryos [78]. At 24 hpc, all the embryos can be found in the isthmus [95]. At 78 hpc about one-third of the embryos are already found beyond the utero-tubal junction, whereas at 84 hpc more than 90% of the embryos have reached the uterus [95]. Embryo implantation occurs between 120 and 144 hpc [96]. Considering the chronology of embryo development some techniques have been developed to recovery embryos at any stage and location.

Embryos can be recovered by non-surgical, *post-mortem* or laparoscopy. In the non-surgical method, $PGF_{2\alpha}$ is administered 50–55 h after mating. It produces the expelling of embryos, located in the oviduct or the uterus, toward the vagina, where embryos can be recovered

Hormone	Dose	Ovulation induction	Ovulation rate	Recovered embryos	Reference
eCG	200 IU	—	19.2	8.8	[21]
	20 IU/kg BW	120 IU hCG	28.7	14.2	[82]
FSH	5 × 9 µg/ml /12 h	1.6 µg buserelin acetate	26.7	21.2	[83]
	6 × 0.5 mg /12 h	150 IU hCG	26.5	21.3	[84]
pFSH	3 × 18 µg/ml /24 h	2 µg buserelin acetate	34.4	—	[85]
rhFSH	3 × 0.6 µg /24 h	2 µg buserelin acetate	16.6	—	[85]

eCG: equine chorionic gonadotropin; FSH: follicle-stimulating hormone; pFSH: porcine FSH; rhFSH: recombinant human FSH; BW: Body weight; hCG: human chorionic gonadotrophin hormone.

Table 2. Examples of superovulation protocols.

without surgery [97, 98]. This procedure has an efficacy of 40% [97, 98]. When embryos are obtained *post-mortem*, the entire reproductive tract is removed after the female is euthanised. The embryos are recovered by perfusion of each oviduct and the first third of the uterus with 5 ml of phosphate-buffered saline containing 0.2% of BSA [33]. This method allows a retrieval rate of 64% [99]. Laparoscopy is a method that allows the recovery of both preimplantation embryos and also of oocytes [100]. If multiple cycles of embryo collection are required, this method is chosen because it guarantees minimal invasion through the use of a small entrance in the peritoneal cavity. However, this procedure has a lower efficacy in primiparous or multiparous females (around 50%), compared with nulliparous females (73%) [99].

Recovered embryos are subjected to morphological grading (**Table 3**, [101–103]); they are usually classified as having 'good quality' when they present homogenous cellular mass and intact zona pellucida and mucin coat [104]. Normal embryos can be maintained under *in vitro* conditions or cryopreserved until transferred. Zygotes have been successfully developed into blastocyst *in vitro* [10, 31, 104–108]. One example of culture media is TCM199 supplemented with 0.1% of BSA and culture is performed in 500 μl of medium layered under paraffin oil at 38.5°C, 5% CO_2 and saturated humidity [109]. However, these culture media do not mimic the uterine environment, and as a consequence, embryos developed *in vitro* have fewer cells and lower intrazonal diameter and mucin coat thickness than embryos developed *in vivo*, which reduces pregnancy rates after embryo transfer [40, 106].

Embryo transfer is performed by surgery (laparotomy) [110, 111] or by laparoscopy [112], into the oviducts or each one of the uterine horns. The use of laparoscopy aims at minimising the invasion site of the reproductive organs and *in situ* manipulation [79]. To guarantee the success of the transfer, a minimum number of embryos have to be transferred, and asynchrony between the embryo development and the recipient female has to occur [65, 70, 113]. On one hand, at least two foeto-placental units are required to maintain the gestation [114, 115], and on the other hand, when the survival rate is high, competition between foetuses can lead to a lower foetal survival [115]. So, the number of transferred embryos recommended per recipients ranges between 10 and 13 for both fresh and cryopreserved embryos [71, 116]. If the number of transferred embryos per donor female is lower than this range, it is possible

Embryo-grade categories[1]	**Score grade**[2]	**Size blastomeres**	**Cytoplasmic fragments**
Good quality	Grade 1	Equal	None
	Grade 2	Equal	Minor
Fair quality	Grade 3	Unequal	None or few
Poor quality	Grade 4	Unequal	Major
	Grade 5	Any	Severe or complete

[1]Embryos are clustered in embryo-grade categories following the Shulman criterion.
[2]Embryos are graded following the Veeck and Maloney criterion.

Table 3. Grading score used for rabbit embryos.

to transfer embryos from two donor females to each of the uterine horns of the recipient female, to ensure the survival of the embryos [71]. In case it is necessary to know the origin of the transferred embryos, it is recommended to perform a caesarean to the recipient female [71]. In general, asynchrony is obtained by artificially inducing ovulation in recipient female between 0 and 6 h before the donor females, for fresh embryos, or between 6 and 12 h before, for *in vitro* cultured or cryopreserved embryos [60, 65, 70, 113]. Besides, an association of the recipient genotype with implantation rate, foetal losses and offspring rate at birth has been documented [70, 71]. The recipient female usually belongs to a maternal line because recipient genotype is crucial in providing an adequate uterine environment to support adhesion, embryo-uterine cross-talk and placental and foetal development to term after vitrified and fresh embryo transfer [70, 111].

Embryo recovery and transfer influences mRNA expression of late blastocyst before implantation and may result in faulty embryonic implantation [117]. Transferred embryos have a lower transcript abundance of the transcription factor octamer binding 4 (OCT4) and higher epithelial membrane protein 1 (EMP1) [117]. OCT4 is a key regulator of the pluripotency maintenance system [118], and the main function of this transcriptional factor is to repress or activate several target genes involved in cell differentiation and early embryonic development [119]. The altered expression of OCT4 in the preimplantation embryo is associated with lower embryo quality [120]. EMP1 is involved in the regulation of cell cycle or cell-cell recognition, and high levels of EMP1 expression have been related to cell differentiation and arrest [121]. Signals involved in cell proliferation and differentiation during gastrulation and implantation events could be disturbed with embryo manipulation.

7.2. *In vitro* fertilisation and intracytoplasmic sperm injection

Oocyte collection and extracorporeal fertilisation represent an important embryo production in rabbits [73]. These embryos are of scientific interest for cloning and transgenesis [73].

Both *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) have been successfully developed [122, 123]. For IVF, oocytes are incubated with sperm for 5 h, and then moved to the embryo culture medium containing TCM199 medium suspended with 1.25 mM pyruvate, 0.1 mM EDTA, 10% FBS, and cultured at 37°C and 5% CO_2 [122, 123]. For ICSI, the micromanipulation is performed in the fertilisation medium on a warm microscope stage at 37°C. Cumulus cells of mature oocytes are removed by hyaluronidase treatment and gentle pipetting. The injection needle used for rabbit sperm is of 5.0 μm inner diameter. After injection, the oocytes were transferred to embryo culture medium [123].

7.3. Cloning and transgenesis

Cloning involves the transfer of a nucleus from a multicellular embryo, foetal or adult cell into an enucleated metaphase II (MII) oocyte [124]. This oocyte has the ability to incorporate the transferred nucleus and support development of a new embryo. Cloning of embryos by nuclear transfer technology (NT) has been developed in several species [125–127]; the rabbit was a pioneering species in NT by introducing embryonic cells into enucleated oocytes, at the

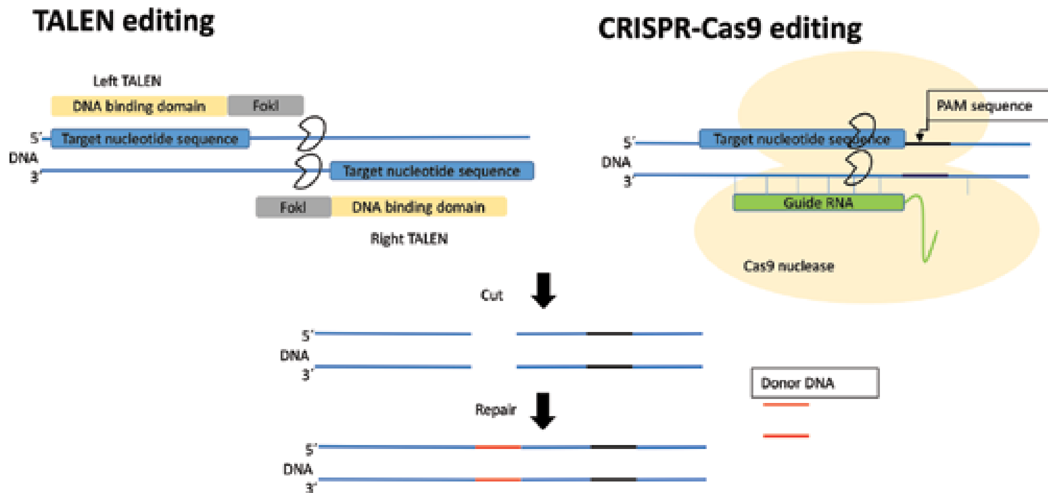

Figure 2. TALEN and CRISPR-Cas9 genome editing systems.

end of the 1980s [128, 129]. However, the use of highly differentiated somatic cells as nuclear donors remained a challenge. It was not until 2002 that live clones were generated from NT with freshly prepared adult rabbit cumulus cell [130]. And later, clones were produced from foetal [131] and adult fibroblast [132].

Overall, rabbit NT efficiency depends on the enucleation of the recipient oocyte, fusion of the transplanted nucleus to the enucleated oocyte, activation of the oocyte and reprogramming of the transferred nucleus. On enucleation, the visualisation of the MII is difficult, because of the presence of dark cytoplasmic granules. To overcome this problem, MII can be detected with low ultraviolet light, which allows the removal of MII and polar body under visible light using an enucleation pipette with a minimal volume of oocyte cytoplasm [133]. Enucleation rates vary from 60 to 90% [134, 135], and the age of the recipient oocyte plays an important role in successful NT [136, 137]. Briefly, for nuclear transplantation, single donor cell is introduced beneath the zona pellucida of the enucleated oocytes by micromanipulators. Electro-cell fusion (3.2 kV/cm, 20 μs and three pulses) is applied to fuse the donor cell with the cytoplasm of the reconstructed embryos [138].

Classically, gene transfer has been carried out by microinjection of DNA constructs into fertilised oocytes or by using viral factors. Notwithstanding, with the progress in molecular technology, embryos have been genetically modified using most recently developed tools including TALEN [14] and CRISPR-Cas9 [139, 140] (**Figure 2**).

Both techniques, cloning and transgenesis, have low applications in livestock production due to problems derived from detection of genetically superior animals and evaluation of the clones and the transgenic animals [141]. Some implications for the use of transgenic rabbits nowadays include to act as bioreactors [142] or model for detailed analysis of spermatogenesis [143], and more recently, to establish embryonic stem cell lines from blastocyst stage rabbit embryos cloned by somatic cell NT [138, 144].

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8. Conclusion

There are clear advantages in the use of rabbit for embryo studies. The embryo development is similar to human, so rabbit is a suitable model for the application of embryo reproductive techniques. Important insights have been developed successfully in embryo recovery and transfer, cryopreservation, *in vitro* fertilisation, cloning or transgenesis. In addition, these findings have been applied to improve rabbit breeding.

Conflict of interest

The author declares that there is no conflict of interest.

Abbreviation list

BSA	bovine serum albumin
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats-associated nuclease Cas9
DPBS	Dulbecco's phosphate-buffered saline
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EMP1	higher epithelial membrane protein 1
FBS	foetal bovine serum
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotrophin
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilisation
LH	luteinizing hormone
MII	metaphase II
NT	nuclear transfer technology
OCT4	transcription factor octamer binding 4
$PGF_{2\alpha}$	prostaglandin F-2α
rhFSH	recombinant human FSH
TALEN	transcription activator-like effector nucleases

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Chapter 8

Intraoviductal Instillation of a Solution as an Effective Route for Manipulating Preimplantation Mammalian Embryos *in vivo*

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Additional information is available at the end of the chapter

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Abstract

Preimplantation embryos of mammals are enclosed by a translucent layer called zona pellucida (ZP), which is composed of glycoproteins. ZP is important for protecting against infection by virus and bacteria, and to prevent attachment of embryos to the oviductal epithelia. Due to the presence of ZP, it has been difficult to transfect preimplantation embryos existing within the oviductal lumen, with exogenous nucleic acids, such as DNA and mRNA. However, intraoviductal instillation of nucleic acids, and subsequent *in vivo* electroporation in pregnant females, enables transfection of these embryos, leading to the production of gene-modified animals. This new method for production of genetically modified animals does not require any *ex vivo* handling of embryos, which has been essential for traditional transgenesis. In this article, we describe recent advances in the *in vivo* transfection of preimplantation mammalian embryos, and also the possibility of simple transfection of these embryos through intraoviductal instillation of a solution, alone.

Keywords: genome editing, GONAD, *in vivo* gene transfer, oviduct, preimplantation embryos

1. Introduction

Ex vivo handling of early embryos, which includes embryo collection from pregnant animals, introduction of genetic material or genetically modified embryonic stem (ES) cells into the isolated embryos, and egg transfer (ET) to a recipient female reproductive tract, has been considered essential for production of transgenic and knock out (KO) animals [1, 2]. The recently

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developed genome editing technology, as exemplified by CRISPR/Cas9 system, requires *ex vivo* handling of embryos, namely, microinjection of the genome editing component into the zygote, or *in vitro* electroporation (EP) in the presence of those materials, embryo culture, and subsequent ET [3–7]. Unfortunately, such *ex vivo* handling of early embryos requires an expensive manipulator, special technicians to handle this machine and perform ET, and preparation of recipient females for ET, all of which are sometimes time-consuming and laborious. If this *ex vivo* handling of embryos is omitted, embryo manipulation, including production of genome-edited animals, would become more convenient.

Preimplantation embryos present within the oviductal lumen would be the most favorable targets for genetic manipulation, since the oviducts themselves can be easily exposed outside the individual, under anesthesia, and manipulation within this organ would be possible under observation, using a dissecting microscope. Genetic material can be introduced into zygotes or cleaving embryos, floating in the oviductal lumen, by inserting a glass pipette through the oviductal wall, and introducing genetic material successfully into these embryos by penetrating the zona pellucida (ZP). If this event occurs, *in situ* production of genetically modified embryos (animals) would be possible.

The ZP is a multilayered porous matrix of glycoproteins that envelopes mammalian oocytes and preimplantation embryos, protecting them from environmental insults, including viral infection, and injury by chemical or physical substances [8]. Therefore, it has been difficult to transfect mammalian oocytes and preimplantation embryos, with the usual transfection methods that have proven useful for somatic cells. Early attempts at gene delivery to preimplantation embryos involved the transfection of ZP-free embryos. However, such embryos are vulnerable, adhesive, and easily damaged [9]. The most commonly method used for penetration of ZP for gene delivery is the pronuclear microinjection using purified DNA [10], or the microinjection of viral elements into the perivitelline space, between ZP and the zygotes [11]. Furthermore, it is possible to transfect mouse fertilized eggs with lentiviral vectors, *via* laser perforation of ZP [12]. *In vitro* EP was found to be effective for incorporation of plasmid DNA and morpholino, into mouse fertilized eggs, after ZP was weakened by a brief treatment with acidic Tyrode's solution [13–15]. This treatment allowed the enhanced uptake of exogenous DNA, and protected the embryos from electroporation damage. Notably, previous reports clearly indicate the need for penetrating the ZP for manipulation of early embryos, and also the *ex vivo* handling of embryos prior to ET. Thus, it may be desirable to perform gene delivery to preimplantation embryos, without handling the embryos *ex vivo,* and if possible, to employ substances that will allow direct gene delivery through penetration of ZP.

In the present article, we first discuss studies that have reported successful delivery of substances to ZP-enclosed embryos *in vitro,* followed by reports on *in vivo* transfer of substances into ZP-enclosed preimplantation embryos, after intraoviductal instillation. Lastly, we describe the recent advances in *in situ* genome editing of embryos, present in the oviductal lumen.

2. Transfer of exogenous substances into ZP intact embryos *via* simple incubation

As mentioned previously, it may be possible to perform gene delivery to embryos if a substance capable of penetrating ZP is used in combination with nucleic acids. For example, Ivanova et al.

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[16] demonstrated that when ZP intact preimplantation embryos from mice and rabbits were incubated in a medium containing DNA-carrying constructs with insulin as an internalizable ligand, (insulin-polylysine)-DNA and (insulin polylysine)-DNA-(streptavidin-polylysine)-(biotinylated adenovirus), the constructs penetrated the ZP and accumulated within each blastomere. Southern blot hybridization revealed chromosomal integration of transgenes in mid-gestational fetuses and in a newborn. The ligand-mediated gene delivery to early embryos can be explained by the following mechanism. Insulin provides delivery inside the cell, while adenoviruses ensure release from the endosomes. They called this type of gene delivery "receptor-mediated gene transfer". According to the authors, the construct containing DNA and insulin, penetrates inside, to accumulate in the peri-nuclear space of the embryos. Munk et al. [17] demonstrated that multiwall carbon nanotubes (MWNTs) could cross the ZP to help the delivery of plasmid DNA into bovine embryos *in vitro*. Exposure of embryos to MWNTs did not affect their viability and gene expression. Interestingly, Joo et al. [18] recently developed a hydrophilic, Cy5.5-labeled organic compound called "VisuFect". When VisuFect is conjugated with poly(A) oligo, this complex successfully penetrates the ZP of the fertilized eggs of various species, including zebrafish, mice, and pigs, suggesting that VisuFect can be used to deliver genome-editing nucleic acids to ZP intact embryos to generate genetically modified animals. Unfortunately, VisuFect can deliver oligonucleotides to embryos, but not larger molecules, such as the plasmid DNA. Probably, genome editing in embryos derived from Cas9-expressing transgenic (Tg) mice [19–25] may be possible when VisuFect is used in combination with a smaller molecule such as guide (g) RNA.

As mentioned previously, EP enables the delivery of exogenous substances to somatic cells and ZP-enclosed embryos. Grabarek et al. [13] was the first to demonstrate that nucleic acids can be efficiently delivered to isolated embryos (oocytes and zygotes) by EP. Recently, Kaneko et al. [26] first demonstrated successful induction of genome editing of the target locus in rat embryos, when they were electroporated *in vitro* in the presence of CRISPR/Cas9-related components (Cas9 mRNA + gRNA). In this case, there was no need to weaken ZP, as Grabarek et al. [13] demonstrated. Since this report, similar success in the production of genome-edited animals has been reported in mice and pigs [27–29].

3. Intraoviductal instillation of a solution

Delivery of liposomally encapsulated DNA directly into the oviductal lumen was first reported by Esponda's group [30, 31]. The purpose of their study was to transfect epithelial cells lining the oviductal lumen, not to deliver genetic materials to preimplantation embryos floating in the oviductal lumen. They found that ~6% of oviductal epithelial cells were successfully transfected. Sato [32] injected a solution containing plasmid DNA [conferring enhanced green fluorescent protein (EGFP) expression] into the oviductal lumen of pregnant female mice on Day 0.4 (~11:00 h; corresponding to early zygotes; the day when the vaginal plugs are detected is designated as Day 0 of pregnancy), and then performed *in vivo* EP of an entire oviduct, in an attempt to transfect zygotes with the exogenous DNA. Unfortunately, the attempt to transfect zygotes failed; only a cellular remnant, probably derived from a part of the zygotes, was found to be fluorescent. In contrast, a maximum of 43% of oviductal epithelial cells facing the oviductal lumen, were fluorescent. It was speculated that the failure of gene delivery to the zygotes may be because the cumulus cells surrounding the zygotes function as a barrier [32].

Figure 1 represents a schematic illustration of the structure of murine oviduct and ovary, on different days of pregnancy; Days 0.4 (**Figure 1a**), 0.7 (~16:00 h; corresponding to late zygotes; **Figure 1b**) and 1.4 (~1d, 11:00 h; corresponding to 2-cell embryos; **Figure 1c**), are illustrated. After mating with males, ovulated oocytes, tightly surrounded by cumulus cells, are transferred to the specific site of the oviduct - the ampulla- following which fertilization occurs at

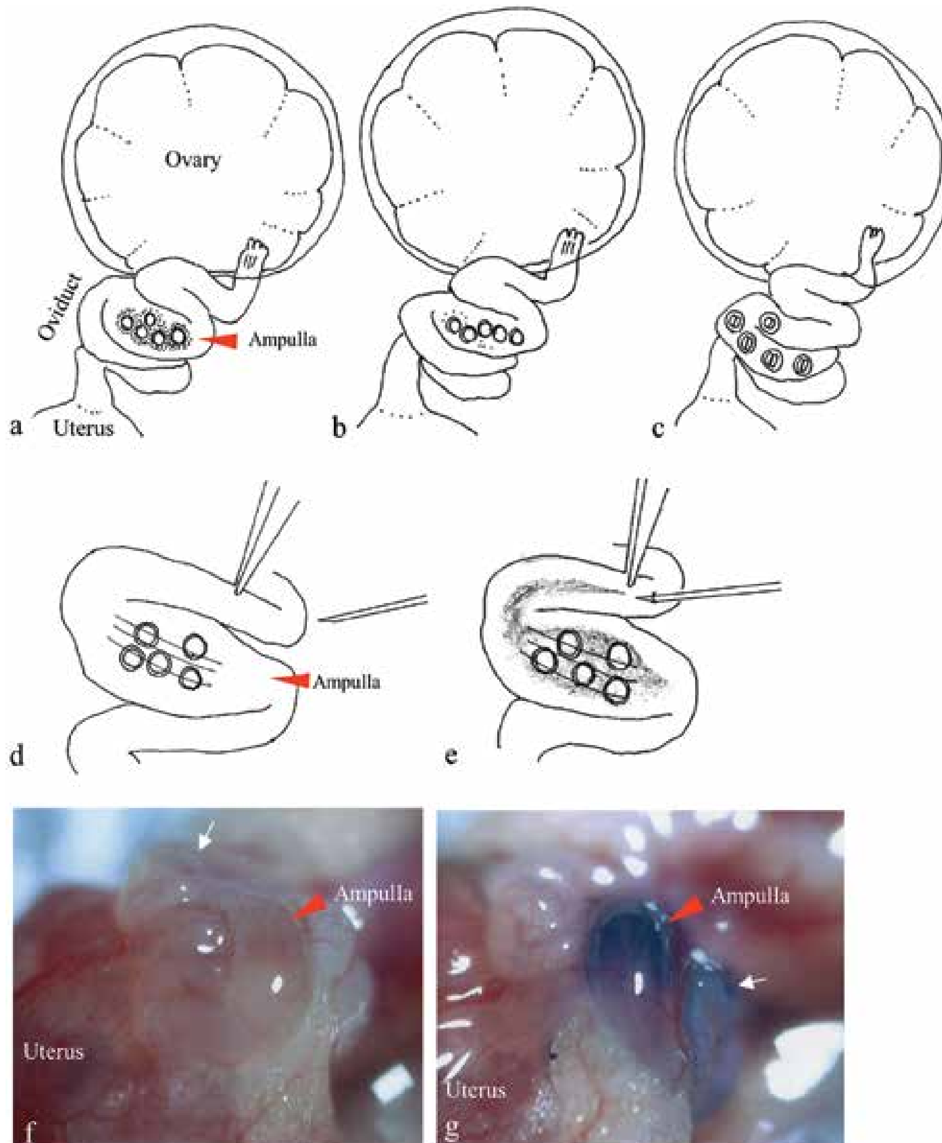

Figure 1. Procedure for the intraoviductal instillation of a solution. a-c: schematic illustration of ovary/oviduct/uterus of females at days 0.4 (a), 0.7 (b) and 1.4 (c) of pregnancy. d, e: schematic illustration of the moment before (d) and during (e) the intraoviductal instillation of a solution through oviductal wall. f, g: Oviduct at day 0.4 before (f) and after (g) intraoviductal instillation of trypan blue-containing solution. Arrows indicate the site through which a glass micropipette was inserted into the oviductal lumen.

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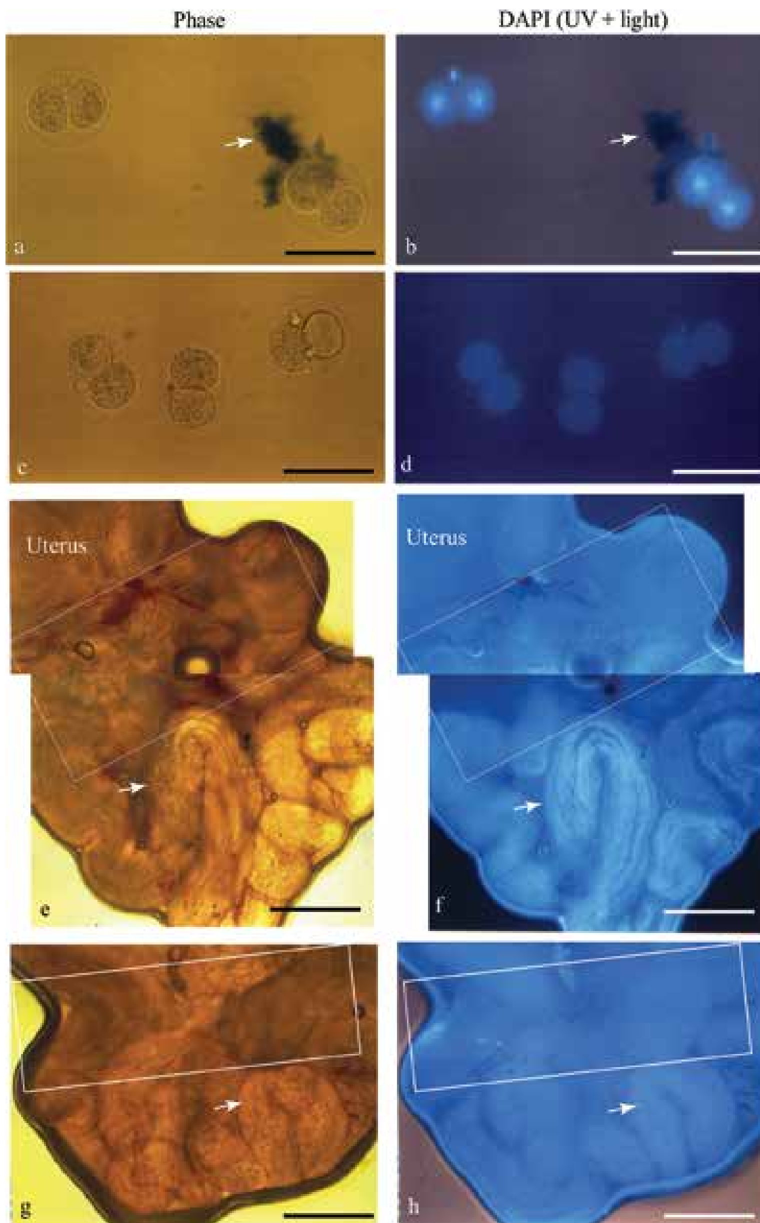

Figure 2. Intraoviductal instillation of a solution containing DAPI and trypan blue. a, b, two-cell embryos isolated from a female 1 day after the intraoviductal instillation of a solution containing DAPI and trypan blue on day 0.4 of pregnancy. Note the presence of bright DAPI-derived fluorescence in the nuclei of 2-cell embryos. Arrow indicates aggregates of trypan blue, which might have formed after introduction into the oviductal lumen. Bar = 100 μm. c, d, two-cell embryos isolated from a female one day after the intraoviductal instillation of a solution containing trypan blue alone, on day 0.4 of pregnancy. No fluorescence is detected in the nuclei. Bar = 100 μm. e, f, DAPI-derived fluorescence in the squashed oviduct from which 2-cell embryos [shown in (a) and (b)] were isolated. Note bright fluorescence in ampulla (arrows), and its neighboring segment of the oviduct. Box indicates the oviductal segment near isthmus. Bar = 250 μm. g, h: DAPI-derived fluorescence in the squashed oviduct from which the 2-cell embryos [shown in (c) and (d)] were isolated. No fluorescence is detected throughout the oviduct, including ampulla (arrows). Box indicates the oviductal segment near isthmus. Bar = 250 μm. a, c, e, g: Photographed under light; b, d, f, h: Photographed under UV and light.

this site. On Day 0.4, zygotes (fertilized eggs) are still enclosed by cumulus cells and exist at the ampulla. On Day 0.7, zygotes are still present at the ampulla, but the ampulla itself, exhibits shrinkage and detachment of cumulus cells from the zygote. On Day 1.4, zygotes cleave to form 2-cell embryos, and are present at the ampulla or the oviductal segment between the ampulla and the isthmus.

In Figure a schematic illustration of the moments before (**Figure 1d**) and during (**Figure 1e**) intraoviductal instillation of a solution is provided. Sato et al. [33] injected a solution containing Hoechst33342 dye, frequently used for vital nuclear staining, into the oviductal lumen of pregnant females at Day 0.4. One day after the surgery, 2-cell embryos were collected from the oviducts for checking the incorporation of the dye in their nuclei. They observed that all the collected embryos had fluorescent nuclei. This means that the dye injected into the oviductal lumen penetrates the ZP, enters the zygotes, and binds to their nuclear DNA. Here, we examined whether another dye 4′, 6-diamidino-2-phenylindole (DAPI), also used for staining of nuclei, can also bind to nuclear DNA of zygotes *in situ*. About 2 μL of a solution containing 1 μg/μL of DAPI and 0.05% trypan blue (used for monitoring successful injection) was injected into the oviductal lumen by piercing the oviductal wall, between the ampulla and the infundibulum (arrows in **Figure 1e** and **f**) of pregnant B6C3F1 (hybrid between C57BL/6 and C3H/He) females on Day 0.4. Successful instillation of a solution can be clearly visualized by the presence of blue dye inside the oviduct (arrowhead in **Figure 1g**). As control, trypan blue alone was similarly injected. When 2-cell embryos are collected from the treated females for inspection of fluorescence, all the collected embryos (6/6 tested) from DAPI-injected females had fluorescent nuclei (**Figure 2a** and **b**); in contrast, the control embryos did not have fluorescent nuclei (**Figure 2c,d**). Furthermore, inspection of DAPI-derived fluorescence in the squashed oviduct revealed the presence of fluorescence in epithelial cells of ampulla and its neighboring oviductal segment (**Figure 2e** and **f**), but no evident fluorescence in the distal segment of the oviduct, near the isthmus (boxed areas in **Figure 2e** and **f**), suggesting that DAPI introduced into the ampulla, might not have been transferred to the oviductal segment near the uterus in a day, after the instillation. The control oviduct never fluoresced (**Figure 2g** and **h**). Thus, DAPI introduced intraoviductally can penetrate ZP-enclosed zygotes and oviductal epithelia facing oviductal lumen.

4. Gene delivery into preimplantation embryos *in vivo*

To our knowledge, successful gene delivery into preimplantation embryos *in vivo* was first reported by Sato et al. [33], who demonstrated the intraoviductal instillation of a solution containing plasmid DNA (conferring EGFP expression) and trypan blue using B6C3F1 females on Day 1.4 of pregnancy (corresponding to 2-cell stage). Subsequently, *in vivo* EP into an entire oviduct was performed using a square-wave pulse generator, T-820 electroporator (BTX Genetronics Inc., San Diego, CA, USA), under the conditions of eight square-wave pulses, with a pulse duration of 50 ms, and an electric field intensity of 50 V. One day after the surgery, 8-cell embryos were collected for checking EGFP-derived fluorescence. About 33% of embryos exhibited fluorescence, although its intensity varied among embryos. However,

when the same procedure was later applied to 2-cell stage embryos using a different electroporator (NEPA21; NEPA GENE Co., Chiba, Japan), all the collected embryos failed to fluoresce (Sato et al., unpublished results). In this case, the ZP appears to act as a critical barrier, preventing substance penetration. In other words, ZP may allow penetration of smaller molecules such as gRNA, but not of large molecules like plasmid DNA. In this context, careful monitoring of EP conditions may be required when larger sized molecules are intended to be introduced into early embryos *in vivo*.

Takahashi et al. [34] employed the method of Sato et al. [33] to induce genome editing in the target gene (*EGFP* cDNA) in EGFP-expressing Tg 2-cell embryos *in situ,* through intraoviductal injection of a solution containing CRISPR/Cas9-related components (Cas9 mRNA + gRNA targeted to *EGFP* cDNA). Inspection of fluorescence under a fluorescence microscope demonstrated that 33% (2/6) of the resultant mid-gestational fetuses exhibited complete loss of EGFP-derived fluorescence, and 33% (2/6) exhibited reduced fluorescence. Sequencing analysis of genomic DNA isolated from these fetuses revealed that no mosaic mutations were detectable in the samples showing complete loss of fluorescence. Mosaic mutations were also detectable in the samples showing reduced fluorescence. Based on these findings, it was found that intraoviductal instillation of a solution containing CRISPR/Cas9-related components, and subsequent *in vivo* EP, enables genome editing in a whole embryo. Thus, Takahashi et al. [34] named this technology "Genome editing via Oviductal Nucleic Acids Delivery (GONAD)".

As mentioned above, GONAD on Day 1.5 results in production of individuals with highly frequent mosaic mutations. Genome editing at 2-cell embryo stage, appears to be the major cause of this problem. To circumvent the issue of mosaic mutations, performing GONAD on Day 0.7, a stage corresponding to late 1-cell embryos, from which cumulus cells begin to detach (see **Figure 1b**), was considered. Ohtsuka et al. [35] examined whether embryos at this stage are suitable for GONAD by injecting a solution containing *EGFP* mRNA (1 μg/μL) and trypan blue (0.05%) into the lumen of an oviduct of pregnant ICR females, since EGFP-derived fluorescence can be easily monitored under a fluorescence microscope. Two days after GONAD, 8-cell embryos were isolated from the oviducts of the GONAD-treated females, and checked for EGFP-derived fluorescence; several of the collected 2-cell embryos were found to be fluorescent. Notably, no or less biased fluorescence was evident in each blastomere among the fluorescent embryos, suggesting low mosaicism in the treated embryos.

5. Genome editing in preimplantation embryos after performing GONAD on day 0.7

Next, we tested whether GONAD can indeed induce specific mutations on the endogenous target locus in late 1-cell embryos. We chose α-1, 3-galactosyltransferase (α-GalT) gene (*GAAT1*) as a target gene to be knocked out. α-GalT is a key enzyme, capable of synthesizing the cell-surface glycoprotein, called α-Gal epitope that is expressed in all mammals, except humans and Old-Word monkeys [36, 37]. Complete KO of *GAAT1* leads to loss of α-Gal epitope expression, which can be easily monitored by staining with α-Gal epitope-specific

lectin called BS-I-B_4 (IB4) [38, 39]. Indeed, cloned porcine blastocysts, after reconstitution with *GAAT1* KO nuclei, lost the reactivity against fluorescence-labeled IB4. However, residual amounts of α-Gal epitope, which may have been derived from maternally accumulated products of α-GalT, were occasionally recognized by the α-Gal epitope-specific lectin in the cloned blastocysts [40].

We designed gRNA (5′-GAGAAAATAATGAATGTCAA-3′) for targeting exon 4 of murine *GAAT1*. A part of the gRNA was synthesized by Integrated DNA Technologies, Inc. (IDT; Coralville, Iowa, USA) as Alt-R™ CRISPR crRNA product. The crRNA and tracrRNA (purchased from IDT) were combined for annealing and then mixed with recombinant Cas9 protein (TaKaRa Shuzo Co. Ltd., Shiga, Japan) to form a complex called ribonucleoprotein (RNP). The final concentrations of the components in RNP were 30 μM (for crRNA/tracrRNA) and 1 mg/ml (for Cas9 protein). GONAD was performed in superovulated B6C3ICR (hybrids between B6C3F1 and ICR) females (10–16 weeks of age) using RNP. Two days later, morula were collected from the oviducts, fixed in 4% paraformaldehyde (PFA) for 1 day at 4°C, and then stained with Alexa Fluor 594 (AF594)-labeled IB4 lectin (hereafter referred to as AF594-IB4) for 1 day at 4°C. About 42% of morulae (5/12) tested, exhibited decreased levels of fluorescence after staining with AF594-IB4, as exemplified by embryo #4 (**Figure 3a** and **b**), suggesting successful genome editing at the target locus, *GAAT1*. Notably, the residual amounts of maternally accumulated α-Gal epitope, appeared to be still discernible in this embryo (#4 in **Figure 3a** and **b**). The remaining seven embryos (including the embryos #1 to #3 in **Figure 3a,b**)

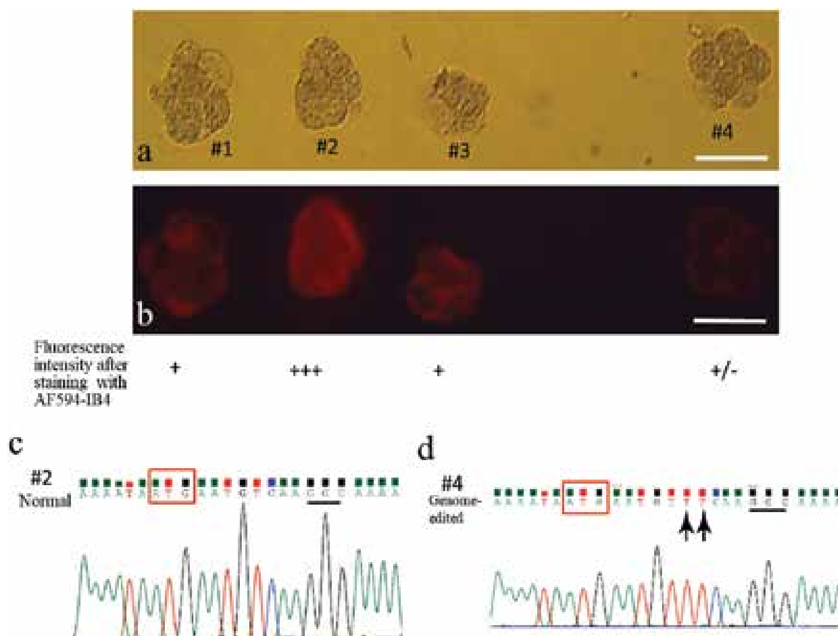

Figure 3. *i*-GONAD-mediated production of genome-edited morulae using RNP, targeted to mouse *GAAT1*. A, b, staining of *i*-GONAD-treated embryos with AF594-IB4. Of the four embryos stained, embryo #4 exhibited highly reduced expression of α-gal epitope. Bar = 100 μm. a: Photographed under light; b: Photographed under UV and light. c, d: direct sequencing of PCR products derived from the *i*-GONAD-treated single embryo, shown in (a) and (b), and corresponding to the embryos #2 and #4, respectively. Boxes indicate the translation initiation codon ATG; underlines indicate PAM.

exhibited reactivity to AF594-IB4, suggesting that they might be unedited or have mono-allelic mutations for *GAAT1* locus. These stained embryos were individually subjected to lysis for genomic DNA isolation, whole genome amplification (WGA), and PCR/nested PCR for direct sequencing, to examine the presence of mutations in the target gene, based on our previous procedure for detecting mutations in the porcine *GAAT1* gene [41, 42]. The primers used were 5′-GCAAATGTGGATGCTGGGAAC-3′ (sense primer)/5′-ACAGTTTTAATGGCCATCTGG-3′ (reverse primer) and nested primers were 5′-TGAATCGAGCAGGTGTTTCAT-3′ (sense primer)/5′- AGGAACACAGGAAGACTGGAC-3′ (reverse primer). The expected size of PCR products was 390 bp (for 1st PCR) and 344 bp (for nested PCR). Direct sequencing of the PCR products demonstrated that, in the embryo showing decreased expression of α-Gal epitope (#4 in **Figure 3a** and **b**), there was an insertion of two nucleotides (AA) (shown by arrows in #4 of **Figure 3c**) above the protospacer adjacent motif (PAM) on the target locus, *GAAT1*. Furthermore, the sequence around the insertion site had no additional peak in the electrophoretogram, suggesting that #4 is a homozygous bi-allelic KO embryo. The amino acid sequence deduced from the nucleotide sequence of sample #4 is Met N V S R E K Stop, which differs from that of the authentic sequence (Met N V K G K V I L L Met L I---) and had premature termination of protein synthesis at seven amino acids starting from Met. On the other hand, embryo #2, showing normal expression of α-Gal epitope, had no mutation on the target region (#2 of **Figure 3c**). Thus, GONAD, using RNP, is useful for inducing genome editing within the target locus in preimplantation embryos.

6. Improved (*i*)-GONAD in mice

As already mentioned in Section 4, Ohtsuka et al. [35] modified GONAD as follows: 1) RNP was employed instead of Cas9 mRNA and gRNA; and 2) *in situ* genome editing within embryos was carried out at Day 0.7 of pregnancy. As a result, they succeeded in obtaining mice with insertion/deletion mutations in their genome at relatively high frequencies (~100%), as well as mice with large deletions in a target gene, and with knock-in of a desired sequence, into a target locus. Furthermore, the mutations acquired through genome editing, in the target locus, were transmitted to the next generation. Therefore, they re-named this improved technology "improved GONAD (*i*-GONAD)". According to Ohtsuka et al. [35], *i*-GONAD is usually performed at ~16:00 from the day of the vaginal plug detection. However, this timing often causes inconvenience for users, such as researchers and technicians. If possible, experiments are done during the daytime, from 9:00 to 16:00. Since the timing for ovulation, and fertilization by sperm, can be shifted by changing the time of gonadotrophin administration, we first examined whether the time at which late 1-cell embryos are obtained may be shifted forward, by administering gonadotrophins, at an earlier time. Adult B6C3ICR females (10–16 weeks of age) were intraperitoneally (i.p.) injected with 5 IU of pregnant mare's serum gonadotrophin (PMSG; eCG) at 11:00, followed by human chorionic gonadotrophin (hCG) of the same dose, 48 h later. Thereafter, the females were allowed to mate with the males. The next morning (~11:00 h; corresponding to Day 0.7 of pregnancy), oviduct/ovary/uterus were dissected and the morphology of ampulla was observed under a dissecting microscope; the ampulla exhibited a tendency of shrinkage (arrow in **Figure 4a**). Flushing of the oviduct resulted in release of cumulus cell-free zygotes (**Figure 4b**). These findings were

indeed consistent with the previous findings of Ohtsuka et al. [35], who observed shrinkage of ampulla and cumulus cell-free zygotes when females, at Day 0.7 of pregnancy, were inspected at ~16:00. Thus, hormone-mediated shift in timing for acquiring late 1-cell embryos was found to be successful.

Administration of higher dose (in this case, more than 5 IU) of gonadotrophins can induce superovulation, but often causes failure to deliver pups [43–45]. Some reports state that the administration of low-dose gonadotrophins (less than 5 IU) facilitates the ovulation of a natural number of oocytes, and successful delivery of pups [46, 47]. This evoked us to suppose that the use of females who have achieved pregnancy after administration of low-dose gonadotrophins and subsequent mating with males would be preferable for *i*-GONAD experiments. To verify this strategy, three adult B6C3ICR female mice (10~16 weeks of age) were i.p. administrated with either 5, 2, or 0.5 IU of PMSG at 11:00, followed by 5 IU of hCG, 48 h later (**Figure 4c**). In this case, light/dark cycle (7:00/19:00) in a mouse room remained unchanged. Thereafter, the females were mated to males (with a ratio of one female: one male). In case of administration of 5 IU PMSG, two of three females had vaginal plug, but failed to deliver their pups (**Figure 4d**). When these females were inspected later, one female was found to

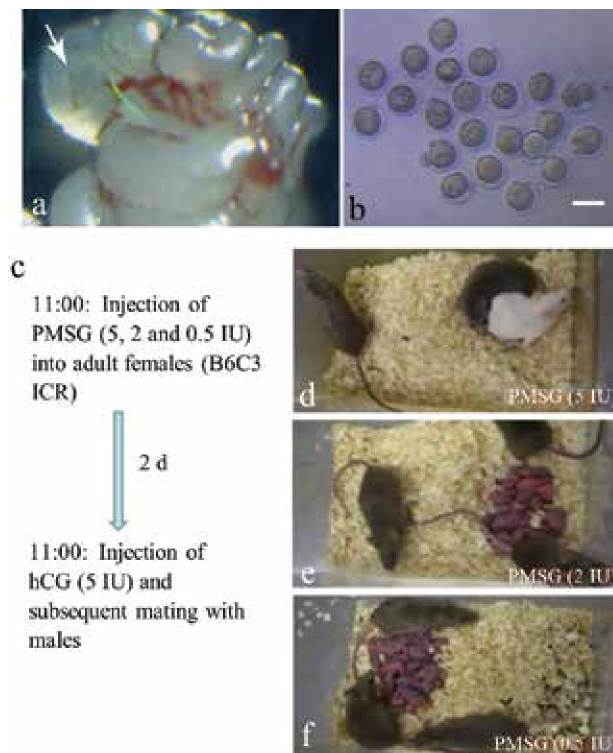

Figure 4. Late zygotic stage embryos can be obtained when superovulation schedule is shifted forward. a, oviduct dissected from a female one day after administration of superovulation-inducing hormones at 11:00. Ampulla (arrows) begins to show shrinkage. b, late zygotes collected from the oviduct shown in (a). Note that the isolated zygotes are already free from cumulus cells. Bar = 100 μm. c, a regime for performing *i*-GONAD towards late 1-cell embryos by administering gonadotrophins at an early time. d-f, birth of pups from females administered varying amounts [5 IU (d), 2 IU (e), or 0.5 IU (f)] of PMSG. Note that females treated with low-dose PMSG (2–0.5 IU) successfully delivered normal number of pups, but those treated with 5 IU PMSG failed to deliver their pups.

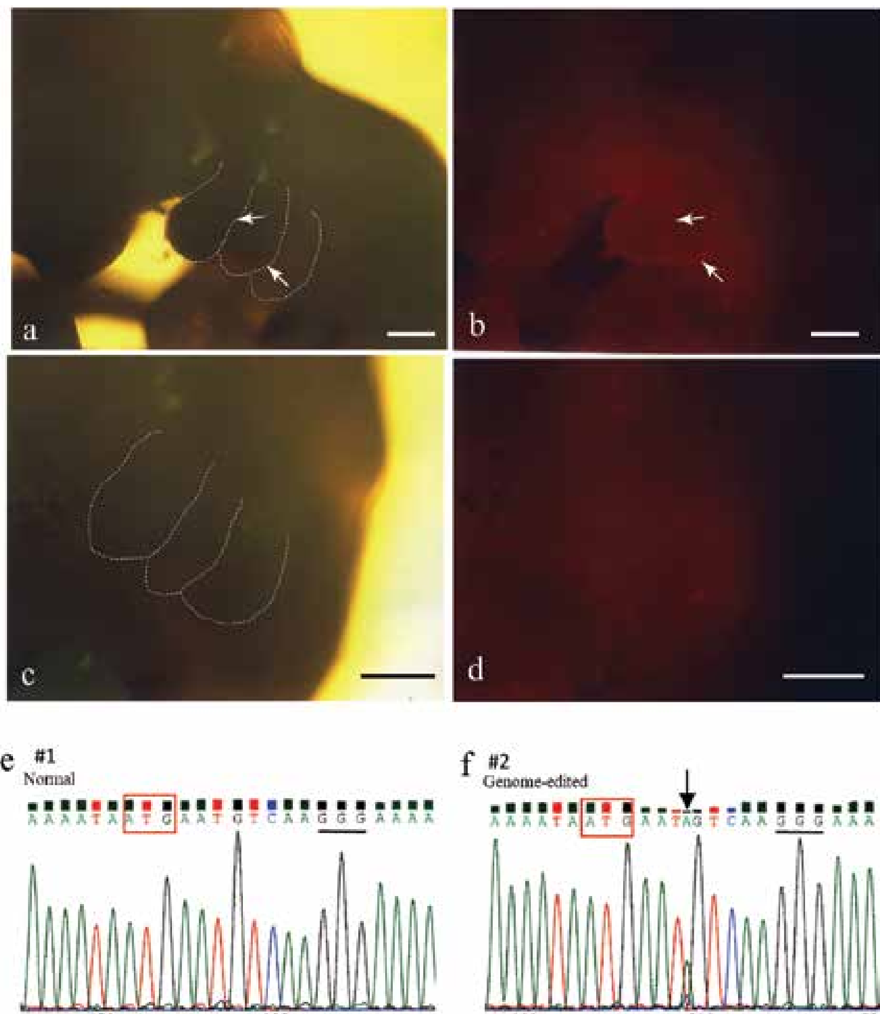

Figure 5. *i*-GONAD-mediated production of genome-edited mid-gestational fetuses, using RNP targeted to mouse *GAAT1*. a–d, staining of *i*-GONAD-treated fetuses with AF594-IB4. One fetus [numbered #1; shown in (a) and (b)] was strongly stained by lectin (indicated by arrows), while the other fetus [numbered #2; shown in (c) and (d)] exhibited highly reduced expression of α-gal epitope. Bar = 500 μm. a, c: Photographed under light; b, d: Photographed under UV and light. c, d: Direct sequencing of PCR products derived from the *i*-GONAD-treated single fetuses, shown in a-d. Boxes indicate translation initiation codon ATG; underlines indicate PAM.

have two dead fetuses in its uterus. In case of administration of 2 IU PMSG, all three females had vaginal plug, and then delivered a total of 23 pups (average: 8) (**Figure 4e**). In case of administration of 0.5 IU PMSG, also, all three females had vaginal plug, and subsequently delivered a total of 25 pups (average: 8) (**Figure 4f**). These findings suggest that administration of low-dose PMSG at 11:00 can lead to natural delivery of normal numbers of pups.

Next, we tested whether genome-edited live mid-gestational fetuses can be obtained by *i*-GONAD, using the same conditions mentioned in Section 4. Adult B6C3ICR females (10–16 weeks of age) were administrated with low-dose hormones according to the regime shown in **Figure 4c**. After administration of hCG, females were mated to males. Next morning,

i-GONAD, using RNP targeted to mouse *GAAT1*, was performed in vaginal plug-positive females at ~11:00. One of the four females had 14 normal and viable fetuses, when inspection was done 11 days after the surgery. All fetuses dissected were fixed in 4% PFA for 1 day at 4°C, and then stained with AF594-IB4 for 1 day at 4°C. Untreated Day 11.5 fetuses were similarly stained with lectin as control. Fluorescence on the surface of the stained fetuses was then inspected under a fluorescence microscope. Ten of the 14 fetuses showed positive and strong staining at the branchial arch (shown by arrows in **Figure 5a** and **b**), like the control fetus. However, the remaining four fetuses exhibited reduced staining for AF594-IB4, at the surface of the branchial arch (**Figure 5c** and **d**). Two of these four α-Gal epitope-negative fetuses, together with another α-Gal epitope-positive one, were subjected to genomic DNA isolation and subsequent PCR analysis, as described in Section 5. Direct sequencing of PCR products demonstrated that the α-Gal epitope-positive samples exhibited unedited sequence of *GAAT1* (**Figure 5e**), like an intact control fetus. In contrast, the samples showing reduced staining for AF594-IB4 (shown in **Figure 5c** and **d**) had one nucleotide (A) insertion above PAM (**Figure 5f**), creating an abnormal truncated protein with sequence MetN S Q G K S N P S Stop. Notably, the presence of a minor single nucleotide "C" overlapped with the inserted nucleotide "A", suggesting that this sample may comprise bi-allelic KO, with different mutated nucleotides. The other α-Gal epitope-negative sample also had mutations (insertion of AA) above PAM in the exon 4 of *GAAT1* (data not shown). From these results, *i*-GONAD performed in females at Day 0.7 of pregnancy, in which natural ovulation was induced after administration of low-dose PMSG, is proven useful for obtaining genome-edited animals (fetuses).

7. Conclusion

Since preimplantation embryos, including embryos at stages of fertilized eggs to cleaved eggs, are floating in the oviductal lumen, it is easy to manipulate them through introduction of substances (such as genome editing-related components, plasmid DNA, viral vectors and chemical reagents) into the oviductal lumen. Particularly, intraoviductal instillation of genome editing-related components and subsequent *in vivo* EP into an entire oviduct are now considered one of the most useful ways to create genome-edited animals (at least in mice and rats) in a convenient manner. Unfortunately, these approaches still require an expensive electroporator apparatus for gene delivery. Rather, in situ transfection of preimplantation embryos without using EP would be desirable. To achieve this, intraoviductal instillation of ZP-penetrating substances (as exemplified by reagents for receptor-mediated gene transfer, MWNTs and VisuFect), together with genome editing-related components, would be one of the promising approaches. If this attempt meets with success, the creation of gene-modified animals, including larger animals such as pigs and bovines, would be accelerated.

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Conflicts of interest

The founding sponsors had no role in the design of the study, collection, analyses, or interpretation of data, writing of the manuscript, and decision to publish the results.

Author contributions

Masahiro Sato designed the study and drafted the manuscript; Masato Ohtsuka provided information of *i*-GONAD-related study and revised the manuscript; Shingo Nakamura critically revised the manuscript.

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This book covers a variety of topics on animal reproduction and reproductive medicine. With evolving technology and a continual increase in knowledge, regarding domestic pets or agricultural animals, new information is available on diverse topics in this broad field. The book contents reflect the individual experience of authors, who developed a number of themes identified as attracting interest in the field. As it is, new opportunities were opened for productive collaboration. We have tried to provide you with current, specialised information that may be useful to students, clinicians and researchers. We hope this book inspires you to embrace these themes, foster the debate on particular topics and may be used as a start-up source for exploring the theriogenology field.

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