PREFACE

Biotechnology of Animal Reproduction is a book concerned with a strategic area of science. All topics on animal production and comparative physiology are very important to aid in the better understanding of areas related to pet companionship, health sciences and food production. Nowadays, humanity is always facing breakthroughs in new technologies, genetics and environmental challenges. This knowledge will lead society to the development of tools that can be used to enhance a balanced way through animal welfare, ecology and production of food. In this context, this book is an attempt at fulfilling the need for gleaned information about the scientific and, most importantly, applied aspects of animal reproduction. Considering the importance of many species that need assisted reproductive technologies, the authors covered the most studied models, including cattle, horses, sheep and others. The authors also exposed the main biotechnologies used in animal assisted reproduction, discussing their indications according to each species to which they are applicable. This book covers topics such as: the reproductive physiology of males and females; production of embryo (*in vivo* and *in vitro*); cryopreservation of embryos; artificial insemination; control of estrous cycle; hormonal treatments; semen analysis; reproductive ultrasonography; *in vitro* culture of ovarian follicles; animal cloning; advances on biotechnology of small ruminants; epigenetics in animal reproduction; equine reproduction and ovum pick up. Over twenty leading scientists from several universities and countries have contributed research to create the first single-source reference on reproductive techniques. It is hoped that this book will be convenient to all categories of people dedicated to animal reproduction, whether they are undergraduate and graduate students, teachers, scientists or practitioners.

Chapter 1 - There are several particularities in the female reproductive system regarding anatomy and physiology between domestic animal species. The female reproductive system consists of the ovaries, oviducts, uterine horns and body, cervix, vagina, vestibule and vulva. The internal structures are supported by ligaments and innervated with autonomic nerves, sensitive and parasympathetic fibers that emit and transmit signals to the organs of the reproductive system. In the ovaries, female gametes are enclosed by follicles with different sizes and at various stages of development. Folliculogenesis and oogenesis are responsible for the formation of the pool of follicles and oocytes that will be consumed throughout the reproductive lifespan. Both processes are dependent of steroid hormones and the gonadal and hypothalamic-pituitary axis, which regulates the female estrous cycle via paracrine and endocrine signals. The cycle is divided into two phases, the follicular or estrogenic phase and

the luteal phase, whose duration vary between species. The secreted hormones promote anatomical changes in the female according to the phase of the cycle. Currently, the interest in researching the mechanisms of folliculogenesis has increased significantly due to the advance in the assisted reproduction biotechnologies. However, several aspects of the regulation of early follicular development and establishment of the ovarian follicular reserve remain unclear. For long years, the concept of formation of follicles during the fetal development and its consumption during the reproductive lifespan of females prevailed. Nevertheless, the concept of the finite pool of follicles/oocytes has been challenged and some new intriguing aspects have been proposed. Evidence of female gamete formation and development in adult ovaries has provided interesting debates about follicle origin and the mechanisms of follicle recruitment and growth. Reports of the presence of multioocyte follicles in adult female ovaries reinforce this polemic issue, because these structures are described in the follicle formation during gestation. Herein, it is presented information of the female reproductive physiology focused on the bovine specie and related to anatomical aspects, estrous cycle and follicles/oocytes origin and formation, as well as advances and new perspectives regarding this issue.

Chapter 2 - Understanding of physiological processes of male reproduction is an important issue for reproductive events in domestic animals, because the physiological basis contributes for advances in reproductive biotechnologies, to the improvement of reproductive efficiency, for treatment of infertility beyond being used as a model for andrological trials in humans. Despite anatomical and physiological peculiarities in different species, the reproductive system of male consists of different organs, which are responsible for steroidogenesis and spermatogenesis. These events are controlled by the central nervous system along with synchronic neuroendocrine actions involving the hypothalamic-pituitary-testicular axis and local growth factors. Spermatogenesis involves the production of spermatozoa in the seminiferous tubules from spermatogonia, mitosis and meiosis, and cell differentiation. All events are dependent of the functionality of Sertoli and Leydig cells beyond gonadotropin and testicular hormonal support. Fertility capacity and the ability to identify subfertility in males have increased importance for future generations in a production system, because potential sire can be mated with hundreds of females using reproductive biotechnologies. However, several factors can affect the male reproductive capacity, such as physiological factors, intrinsic or extrinsic reproductive conditions, health, nutritional status and sexual behavior. Studies on male reproductive physiology have been performed to improve the reproductive performance and better understand events related to different phases of reproduction. These researches have stimulated advances, such as improvement of fertilization capacity of spermatozoa, use of frozen semen and sperm sexing to control sex ratio, enhancing the use of the sire. Herein, we approached a theoretical reference to clarify some aspects of male reproductive physiology towards organs function and hormones and to present advances on this area.

Chapter 3 - Artificial insemination is considered one of the oldest biotechnologies of animal reproduction, which is frequently recommended in order to increasing reproductive efficiency and genetic improvement of cattle. This biotechnology has many advantages compared to natural breeding such as health benefits (control of infectious diseases), genetic improvement (use of genetically improved sires) and improvement of reproductive, zootechnical and economic features of herd (standardized management and animals). This biotechnology also enables a large number of offspring from a single sire, increasing the

dissemination of genetically improved sires. Furthermore, it is an essential tool for other reproductive biotechnologies such as timed artificial insemination, resynchronization of estrous or ovulation, multiple ovulation and embryo transfer, and *in vitro* embryo production. The use of artificial insemination reduces the cost of purchase maintenance of animals, because is not necessary to keep sires on the stock. In this context, semen cryopreservation is a biotechnology which contributes to dissemination of artificial insemination in many domestic animal species, because sperm can be stored for a relatively low cost on the stock. In addition, pharmacological control of the estrous cycle is other strategy that has also contributed to the dissemination of artificial insemination, because the use of hormonal protocols for estrous synchronization enabled management and insemination of large number of females. Therefore, the aim of this chapter was to present the main points of reproductive biotechnologies, mainly the technical parameters, advantages and influencing factors, and to discuss practical strategies to improvement of herd.

Chapter 4 - In cattle and horses, ultrasonography is an important diagnostic tool, particularly for evaluation of reproductive system. Among the many advantages that this technology offers, there is easily and practicality of its application, non-invasive examination and mainly the accuracy of evaluation that allows a detailed real-time examination. Ultrasonography has also contributed to a better understanding of physiological bases and the use of reproduction biotechnologies such as artificial insemination, estrus synchronization, superovulation and *in vitro* embryo production. In cattle, ultrasound can be used to evaluate the reproductive system; for diagnosis of some diseases of the ovaries, uterus, testis and accessory glands; and as an auxiliary tool in the breeding management or in the implementation of reproductive biotechnologies such as early pregnancy diagnosis for estrus resynchronization, to evaluate embryonic losses, fetal sex identification, to evaluate the puberty occurrence in heifers, determine the healthy condition in embryo donors and to assist ovum pick-up procedures for *in vitro* embryo production. In horses, the ultrasound examination is an excellent tool for reproductive evaluation of healthy or pathological conditions (ovarian and uterine), in the identification of cyclicality, in the follicular control for artificial insemination, in early pregnancy diagnosis, diagnosis and management of twin pregnancy, monitoring of pregnancy development, fetal sex identification, and aiding in some biotechnologies applied to mare, for example, the ovum pick-up. Considering the benefits and advances that ultrasound provides for management and biotechnology breeding, this chapter will aim to provide clearly and concisely some topics about the main practical applications (technique of ultrasonography) related to breeding of cattle and horses.

Chapter 5 - Sperm fertility is considered one of the main factors for successful breeding of domestic animals, either through natural mating or after using reproductive biotechnologies. Kinetics (motility and sperm trajectory) and the morphological characteristics of spermatozoa evaluation are essential for ejaculate analyses and are correlated with male fertility. Due to technical and scientific progress and the need to ensure the maximum fertility potential of sperm cells, several semen evaluation methods have been established over time. This analysis aims to identify key factors for semen quality, using sperm trial parameters and obtain the most accurate results, which can be correlated with fertility, infertility or subfertility. Subjective semen assessment by optical microscopy was one of first methods to evaluate sperm cells and is still the most commonly used method. However, due to its subjectivity (high variability in results), other methods such as computer-aided semen analysis (CASA) are greatly required. Semen analyses using CASA systems

associated with flow cytometry have reduced the subjectivity of evaluations, increasing the safety and speed of obtaining data. In addition, these methods have high potential for clinical applications, mainly due to the significant correlation between sperm motility characteristics and *in vitro* or *in vivo* fertilization. However, their use is still limited due to equipment costs, data validation requirements, quality control and analysis standardization, especially between laboratories and research centers. Recently, molecular biology studies, such as genomics and proteomics applied to sperm cells and seminal plasma, have also contributed as tools for seminal assessment and identification of breeding males with improved fertility potential. In this context, the aim of this chapter is to provide a practical approach to the main aspects related to different methods of semen analysis applied to domestic animals, in addition to their advantages, disadvantages and the factors that can influence each type of evaluation.

Chapter 6 - The *in vivo* embryo production is characterized by the superovulation of genetically superior donors and the transference of the obtained embryos to female recipients. The Multiple Ovulation and Embryo Transfer (MOET) technique aims to produce a much higher number of offspring than would normally be possible by natural breeding methods during the productive life of a cow. This chapter was prepared to allow a clear and succinct approach to this issue with an updated review about the several procedures involved with MOET in cattle. At the end of this chapter, the reader will have a general knowledge of this technique, from the physiology related to this biotech to alternatives that can enhance the efficiency of the results. First of all, it is essential to consider embryo technologies as instruments at the service of livestock. The *in vivo* embryo production is a biotechnological tool available for the genetic improvement, whose benefits are strictly dependent on the observed criteria in its usage. The success of MOET will depend on the expertise of those involved, the stringency of the selection of sires and donors, the efficient management of the recipients, the experience in embryo handling and a frank dialogue of the pros and cons of this biotechnology.

Chapter 7 - Ovum pick-up (OPU) has been the best choice for *in vivo* oocyte recovery, widely used for *in vitro* embryo production (IVEP) in cattle. Prior to the advent of ultrasonography, there were several methods of obtaining oocytes from live animals, such as laparotomy, laparoscopy, and colpotomy. However, the risks of peritonitis, evisceration, and adhesions created an important limitation. The first report of oocyte recovery by transvaginal ultrasonography in cattle proved that the technique was simple and appropriate and allowed the collection of oocytes several times from the same animal. When OPU is performed smoothly, there are minimal donor-related risks. The increase in embryoproduction by *in vitro* procedures, compared to that of ET, has made many breeders also choose IVEP to produce top-line calves more quickly. IVEP is used in several countries, but Brazil has achieved a position of prominence due to the number of embryos produced by this technique because of the size of the Brazilian herd, around 200 million head, which mainly consists of *Bos indicus* cattle. Those animals tend to naturally have greater numbers of follicles recruited per wave compared to cows of European breeds. To perform OPU, the authors consider many aspects (vacuum pressure and the needle used) as well as biological factors (donor, hormone stimulation, OPU timing and frequency, and operator experience). This chapter details OPU fundamentals as well as aspects related to methodology, equipment, and operator performance in an effort to achieve greater reproductive efficiency in each female animal.

Chapter 8 - *In vitro* embryo production (IVEP) is an assisted reproductive technology used to spread high quality genetics, considering that both male and female gametes donors

can be selected in order to optimize both meat and milk production chains. Furthermore, this biotechnology has contributed to multiply high-value animals that have died or acquired different kinds of infertility as a consequence of other diseases. IVEP has also become an important study model of physiological processes that occur *in vivo*. However, some of the oocytes are not able to develop into viable embryos. Despite the efforts of researchers to simulate in the laboratory the events that occur in oocytes and embryos' microenvironment, the current yield of blastocysts at the end of the production process is not higher than 30 to 40%. During the embryonic development period, significant events occur, such as cleavage, major genome activation, aggregation and compaction of blastomeres and differentiation into trophoblast or embryoblast. All of these events can be profoundly affected by a variety of intrinsic and extrinsic factors such as inorganic ions, nutrients and metabolic waste in the culture media, gas concentration and luminosity, among many others. However, some of the biochemical and hormonal regulations governing the interaction with the environment are not completely known, preventing improvements of the outcomes when compared to the rates obtained with the *in vitro* technique. Therefore, in order to understand and mimic the *in vivo* maturation of gametes, as well as fertilization and early embryonic development, it is necessary to elucidate the mechanisms involved in those many physiological processes. Such understanding could provide greater excellence, standardization and lower costs to the technique, improving its application either in a large-scale or as a tool for other assisted reproductive biotechnologies.

Chapter 9 - In bovine embryo production programs, the number of produced embryos often exceeds the number of available embryo recipients. Embryo cryopreservation is a technique of preservation in liquid nitrogen (LN) that enables the storage of embryos for extended periods without damaging their viability. The two main techniques for cryopreservation of bovine embryos currently used are slow freezing (also known as conventional freezing) and vitrification. The slow freezing technique is widely used for cryopreservation of *in vivo*-generated embryos and enables the achievement of pregnancy rates similar to those obtained after the transfer of fresh embryos. It is considered a simple and practical technique; however, depends on the acquisition of the freezing equipment. Vitrification, in turn, is more suitable for cryopreservation of *in vitro*-produced (IVP) embryos, which are more sensitive to the damage caused by low temperatures than their *in vivo*-produced counterparts. Nevertheless, the warming process of the embryos after vitrification is considered laborious and time-consuming, precluding the application of this cryopreservation method on a large scale. The current challenge consists of developing a protocol for cryopreservation of IVP embryos that provides efficiency, convenience and low running cost.

Chapter 10 - The present chapter describes the procedures associated with assisted reproduction in small ruminants, including estrus synchronization, semen technology and artificial insemination, *in vivo* and *in vitro* embryo production technologies, cloning and transgenesis. All these reproductive biotechnologies are currently applied at different levels of the production pyramid as part of livestock improvement programs and/or genetic marketing strategies, and their application is expected to gain market penetration as procedures continue to be optimized for higher efficiency and increased cost-benefit equation.

Chapter 11 - Over the past decade, the expansion of the equine industry has promoted an increase in the global population of this species, reflecting significant progress in the equestrian industry. Increased prominence and economic development of the sector has been

noted, with respect to the production of equines endowed with high genetic potential and economic value and the creation of related jobs and incentives. The breeding of horses depends on reproductive biotechnologies that result in improvements in the genotype, phenotype, and performance of animals. Therefore, applied research on this species has become essential for continued progress. Understanding the basics of equine reproduction in the mare (anatomy, reproductive physiology, seasonality, estrous cycle in the absence of pregnancy, hormonal manipulation, different types of mare management, pregnancy diagnosis, fetal sexing, and alternative reductions of twin pregnancies) and gaining further knowledge of the peculiarities of semen handling is essential for the implementation of advanced reproductive management. In addition, one can utilize biotechnological applications in equine reproduction, including artificial insemination (AI) with fresh, chilled, and frozen semen, embryo transfer (ET), intracytoplasmic sperm injection (ICSI), and gamete intrafallopian transfer (GIFT). These biotechnologies help to overcome barriers and assist in gaining a deeper understanding of the various events involved in equine reproductive physiology. In this chapter, we discuss the theoretical and practical aspects of management and reproductive biotechnologies in equines.

Chapter 12 - The *in vitro* culture of preantral ovarian follicles is a technique that has been attracting growing interest, because it aims to recover oocytes from follicles that otherwise would not reach the final stage of development *in vivo*, because of the physiological process of atresia. Preantral ovarian follicles (PAOF) are formed during fetal stages by folliculogenesis and represent 90–95% of the ovarian follicular population. However, only 0.1% of these follicles reaches the pre-ovulatory stage and become fertile oocytes that can be fertilized. Thus, the reproductive potential of the mare is underutilized. The biotechnology of PAOF culture allows *in vitro* recovery, development, and maturation of these follicles, thereby facilitating the production of animals of high genetic value and endangered species. PAOF is also a model for the study of poorly understood mechanisms of the initial stages of folliculogenesis that facilitates *in vitro* investigations into the effects of drugs on reproductive physiology. This biotechnology is focused on aspects of *in vitro* culture, preservation, and transportation of the follicles, and cryopreservation and recovery of the oocytes. In this chapter the biotechnological fundamentals of *in vitro* culture of PAOF, aspects related to its application in animal reproduction, and its current situation will be discussed.

Chapter 13 - Since the first adult animal, Dolly the sheep, was cloned from a somatic cell, more than 20 different species of animals have been cloned by somatic cell nuclear transfer (SCNT). Animal cloning has many applications including the replication of genetically superior farm animals, rescuing of endangered species, and the production of genetically modified animals for research. However, the efficiency of animal cloning by SCNT remains very low. This is likely due to insufficient nuclear reprogramming leading to altered gene expression during embryo and fetal development. However, recent attempts to improve nuclear reprogramming have significantly improved animal cloning efficiency, particularly in laboratory species. This indicates that more effective protocols for SCNT cloning may also be developed for large animal species, including livestock. This chapter presents an overview of animal cloning history, applications, technical approaches, limitations and proposed alternatives for improving SCNT cloning efficiency.

Chapter 14 - The mechanism by which totipotent cells containing the same genetic information differentiate into more than 220 cell types during conceptus' development is known as Epigenetics. This area of genetics studies the changes in gene expression and cell

phenotype that are heritable through cell divisions and controlled by factors others than the primary sequence of DNA. Epigenetic mechanisms such as DNA methylation and histone modifications play a key role in gametogenesis and embryogenesis, in a way that failures in these mechanisms may lead to subfertility, infertility and embryonic death. DNA methylation is considered a repressive mark and is related to gene silencing when located at the promoters of genes. This epigenetic mark is reprogrammed twice during gametogenesis and early embryogenesis of mammalian species and plays an important role in genomic imprinting and X chromosome inactivation. Histone post-translational modifications can either inhibit or stimulate gene transcription and are also greatly remodeled during embryonic development. Besides DNA methylation and histone modifications, chromatin remodelers, histone variants and noncoding RNAs are also known to modulate gene expression of gametes and embryos, therefore contributing to fertility and proper embryo development. Researches have been targeting a better understanding of the epigenetic mechanisms and the opportunity of manipulating epigenetic events, in order to improve the efficiency of assisted reproduction techniques such as *in vitro* embryo production (IVEP), cloning, trangenesis and intracytoplasmic sperm injection (ICSI).