PHARMACOLOGICAL MANAGEMENT OF THE OVARIAN FUNCTION

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Summary

Several regimens are available for pharmacological intervention to control estrous cycle in livestock. Manipulation of ovarian function is possible only through a precise understanding of physiology and endocrinology that must be also integrated with the knowledge of reproductive pharmacology. Development of antral follicles (>2 mm) in ruminants occurs in a wave-like pattern where each wave includes periods of emergence, growth, dominance and atresia or ovulation. This phenomenon is controlled by neuroendocrine pathways involving gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogens, inhibin, progesterone and prostaglandin (PG) $F_{2\alpha}$. Thus, administration of these hormones has shown a tremendous impact on luteal control, follicular development and ovulation. Current treatments for synchronization of the ovulation in livestock females are focused in reaching acceptable pregnancy rates avoiding estrus detection (i.e. fixed-time artificial insemination; FTAI). This involves the control of follicular dominance, induction of a new follicular wave, control of progesterone levels and synchronization of ovulation. In relation to superovulation and embryo transfer, current simplified protocols achieve the control of follicular development to initiate FSH treatments at the emergence of a follicular wave, as well as allow the synchronization of ovulation, both without the need of estrus detection in donors and recipients. In summary, current approaches to exogenous control of corpus luteum and follicular development allows the application of assisted reproductive technologies such as artificial insemination, superovulation and embryo transfer programs in large-scale systems avoiding estrus detection, simplifying animal handling, diminishing the variability of the response, and improving the overall results.

1. Introduction.

Increasing efficiency in reproduction is usually a common objective for producers, practitioners and researchers working with livestock around the world. Throughout last decades of the 20th century, several technologies have been developed to increase multiplication of valuable genetics, which in females includes estrus synchronization for artificial insemination, ovarian superstimulation for *in vivo* produced embryos, follicular puncture and *in vitro* production of embryos, embryo transfer and related technologies as cloning and transgenesis. Most, if not all of them, involves the control of the ovarian function, which currently represents a major area of study in ruminants.

Control of reproduction in the female requires a precise knowledge of ovarian physiology, considering also the importance of an applied perspective for the development, improvement or simply implementation of reproductive biotechnologies. In the last years, tremendous amount of information has been reported in scientific journals and related media, overflowing the ability to access, prioritize, summarize and incorporate it by students, practitioners or even by researchers. This chapter is written with the objective to integrate -in a comprehensive manner and with a practical

perspective- the main information regarding both ovarian physiology and pharmacological approaches to its control in ruminants.

2. Ovarian Physiology.

Ovarian physiology is briefly described for a better understanding of its relationship with the different hormones that can be used to control reproduction. Ovarian function in ruminants begins early during fetal development, continues from birth during the juvenile phase and it is present throughout adult life. Reproduction is based on a sequence of events in which the ovary is one of the determining organs involved in two main functions, a) production of gametes, and b) production of hormones that act at different levels of the organism. The follicle is the functional unit of the ovary specialized in the development and maturation of the oocyte. The endocrine activity is carried out by both the follicle and the corpus luteum. To understand the ovarian function related to gamete production as well as endocrine activity, current information about the oocyte, follicle and corpus luteum is briefly summarized below.

2.1. Oocyte Development.

Oocyte development begins during the embryo stage of the female and continues throughout her reproductive life. The first phase takes place early in the fetal life with mitotic division of the oogonia. Previously, the primordial germ cells act as stem cells source for oogonia and oocyte differentiation. These cells are originally derived from the inner cell mass of the blastocyst and during the process of gastrulation are differentiated in primordial germ cells than then proliferate in the yolk sack initiating a process that results in the migration to the gonad and the differentiation of the ovary. Although, the notion that these primordial germ cells are the only source of adult germ cell in the female, and that the last mitotic division of oogonia occurs during the fetal life have been recently challenged; the general consensus is that most of the oocyte population that will be used during the females reproductive life is defined before birth. The oogonia are formed when the embryo is approximately 35 days old and undergo mitotic division from day 50 to day 130 of gestation in cattle. At the beginning of the second third of fetal life, a process of oogonial degeneration begins, and more than 60% of cells die before birth.

A second phase of development begins at the end of the first third of fetal life when oogonia enter meiosis and differentiate into primary oocytes. At the same time, other phenomenon occurs outside the nucleus, like the formation of the primordial follicle that includes a series of changes on oocyte growth and differentiation of the surrounding cells. The oocytes remains arrested in prophase I for a prolonged period from late fetal life to puberty. On the other hand, during this period the follicles continue growing and regressing (see folliculogenesis below), but the oocytes resume meiosis only in adult females after the preovulatory LH surge following the estrus.

Resumption of meiosis and maturation of the oocyte is triggered by the preovulatory LH surge and generally takes place just before ovulation. As result, the oocyte extrudes a set of chromosomes to become haploid and the first polar body is evident at ovulation. The oocyte is arrested again at metaphase II of meiosis acquiring the ability to be fertilized.

Finally, after the sperm-oocyte fusion, the oocyte development ends with the second meiotic division extruding the second polar body and leading to the formation of the haploid female pronucleus. In summary, oocyte development is in close interrelationship with follicular development and its fate is dependent on follicular atresia or ovulation. Thus, in a continuous manner throughout life, withdrawal of an oocyte quota from the ovary related to follicular differentiation will occur, in a process known as folliculogenesis.

2.2. Folliculogenesis

Folliculogenesis begins early in fetal life, coincident with the initiation of meiosis in the oocyte. This process conducts the oocyte in an irreversible progression to ovulation or atresia. Atresia occurs continuously from fetal life to the end of life, even during the estrous cycle and anestrous prepuberal period, pregnancy, postpartum or seasonal anestrus. In contrast ovulation occurs periodically, only once in each estrous cycle. In ruminants more than 99% of the follicles undergoes atresia and less than 100 follicles and oocytes are conducted to the fertile pathway. Thus, ovarian manipulations like induction of ovulation for artificial insemination, superstimulation for embryo transfer, and follicular puncture for *in vitro* embryo production simply attempt to save or recover oocytes from follicles that otherwise will undergo atresia and get lost.

Follicles are classified according with their morphology as primordial, preantral and antral follicles that differ in their size, complexity and responsiveness to gonadotropins. The preantral follicles stages have been classified in a number of ways with the objective to simplify its understanding (i.e. primary, secondary, small preantral, large preantral, etc.). As alternative or complementary classification, follicles are named as gonadotropin-independent (primordial, primary and transitory or committed follicles), gonadotropin-responsive (preantral and small antral follicles), or gonadotropin-dependent (medium and large antral follicles).

Folliculogenesis begin with primordial follicles (i.e. gonadotropin- independent) that are surrounded by a single layer of flattened pregranulosa cells. Follicle size is around 40 µm and oocyte size is 25 µm in diameter, being still meiotically and developmentally incompetent. In the first transition from primordial to primary stage, granulosa cells population increase and become uniformly cuboidal. Primordial follicles constantly move from a non-growing pool to enter a growth phase, but the transition from the one primordial follicle to primary stage can be very long. Once follicles have left the pool of primordial follicles they are transitory or 'committed' to gonadotropin-independent growth. As a follicle grows, the oocyte develops and granulosa cells increases in number. At this stage follicular size is about 100 µm and oocyte is about 50 µm in diameter.

Preantral follicles are usually named as small and large preantral follicles. FSH receptors are present in granulosa cells, the oocyte also increases its complexity, and the *zona pellucida* (a glycoprotein membrane surrounding the plasma membrane of the oocytes), absent in primordial follicles, is deposited. Proliferation of the granulosa cells continues forming several layers of cubical cells, the theca interna is differentiated and preantral follicles and oocytes grow. While this stage progress, large preantral follicles

are influenced by FSH and LH and are now named as gonadotropin-responsive follicles, with approximately 150 µm and 70 µm in diameter for follicles and oocytes, respectively.

The next phase begins with the appearance of a cavity or *antrum* into the follicle. Antral follicles are present from 0.1 mm and reach its maximum diameter in adult females being 5-8 mm in sheep, 6-10 mm in goat, and 12-20 mm in cattle. Antral follicles are present at birth and its number increases during prepuberal phase and remain more or less stable until the senescence. The final growth of medium and large follicles is detected after birth. As follicle size increases, there is a correlative increasing in oocyte diameter until the follicle acquire 2-3 mm and the oocyte reaches a maximum size of approximately 120 µm. The growth rate of the antral follicles is low at the beginning of this process requiring two weeks to reach 0.5 mm and antrum appearance. After that, the growth rate increases and follicles larger than 1-2 mm grow about 1-2 mm per day. With this size, antral follicles are closely dependent of the support of FSH and LH, which acquires particular interest for hormonal control of follicular development like synchronization or superovulation treatments. The information achieved with the use of ultrasonography to study development of antral follicles greater than 2 mm represents one of the most significant contribution of the lasts decades. The following section is focused on the final stage of folliculogenesis, which can be hormonally manipulated and where several reproductive technologies are applied.

2.3. Antral Follicle Development in Wave-Like Patterns

Real-time B-mode ultrasonography is nowadays widely used for the individual and frequent study of antral follicles larger than 2 mm. Prior to the advent of ultrasonography in the late 1980's, Variations in ovarian follicular size on given days of the estrous cycle were studied from slaughterhouse ovaries with conflicting reports. The predominant theory was that follicles were continuously emerging in the ovary until one of them reached ovulation after LH peak. None organized pattern of growth had been clearly demonstrated in these years.

However, Rajakowski proposed in 1960 the theory that follicular development occurs in two waves during the estrous cycle in the cow. The wave hypothesis was further supported by studies using follicle marking and cauterization techniques, or by evaluating the number of follicles at different stages of the estrous cycle. However, in 1987 the use of real-time ultrasonography provided definitive evidence, by three different research groups, of the existence of waves of follicular development during the estrous cycle in cattle. In the 1990s this phenomenon was also demonstrated in sheep and goats, as well as other species.

Follicular waves are characterized by a group or cohort of follicles that are recruited at the same time and are visible on the ovary at 3-4 mm in diameter (emergence). One of them (or usually two in goats and some breeds of sheep) is selected to grow beyond the rest (selection). This follicle grows during some days while the other small follicles undergo atresia (dominance). The dominant follicle continues growing to reach preovulatory size in the ovulatory wave (ovulation occurs), or it regresses showing atresia losing its dominance in non-ovulatory waves (regression). After the dominant

follicle reaches its maximum diameter and either ovulates or regresses, it loses its dominance and a new cohort of follicles are recruited and a new follicular wave emerges (follicular turnover).

Two or three (sometimes four) follicular waves occur during the estrous cycle in cattle. The most commonly found pattern in sheep and goats is three and four follicular waves, respectively, with a wide range from two to five waves per estrous cycle in both species. There is a common trait in all of these species: the first follicular wave emerges, on average, on the day of ovulation (Day 0 of the ovulatory cycle). In cattle, the following waves emerge on Day 10 in females with two follicular waves; and on Days 9 and 16 in females with three follicular waves. In sheep and goats, with the exception of Wave 1 the following waves emerge at variable time. By knowing the moment of ovulation it is possible to estimate the emergence of the first wave of the cycle (or Wave 1); however the time of emergence of Waves 2 and 3 is highly unpredictable. This information is a key factor for the control of follicular dynamics and it will be addressed later. Follicular waves occur not only during the estrous cycle, but also during the seasonal anoestrus in small ruminants and postpartum anestrus in cattle, during the prepuberal period, and during pregnancy. Thus, follicular development in a wave-like fashion seems to be present throughout life in the ruminant females.

Follicular wave dynamics are absolutely dependent on FSH and LH support that is influenced by other ovarian and metabolic hormones. In ruminants, there is enough evidence that each wave is preceded by a surge in plasma FSH concentrations. FSH reaches its maximum value when the largest follicle size is about 5 mm in cattle, and then decrease 3 days before deviation among largest and second largest follicle, which occurs at 8 mm in *Bos taurus* cows. Systemic action of estradiol and inhibin-A are both responsible for FSH declination. The dominant follicle produces steroidal and non-steroidal products (inhibin, follistatin, activin and other growth promoting and inhibiting factors) that act systemically, locally or both to suppress the development of the subordinate follicles and prevent the emergence of a new follicular wave. This suppression seems to be acting both via inhibition of plasma gonadotropin concentrations and also reducing sensitivity to FSH. In this sense, as mentioned above, plasma FSH concentrations have been shown to decrease coincident with the apparent moment of selection of the dominant follicle, and exogenous FSH administered at the time of follicle deviation prolongs or rescue the development of the subordinate follicles, which is normally observed in treatments for superovulation.

The effect of FSH on follicular development is possible by FSH receptors present in granulosa cells. However, when FSH declines the dominant follicle acquires alternative gonadotropin support from LH, which clearly distinguishes it from subordinated follicles. Only those follicles that are able to grow under low concentrations of circulating FSH will go ahead. The future dominant follicle acquires LH receptors in granulosa cells just before FSH completely decline, at approximately 8 mm in cattle and 4 mm in sheep and goats. This shift from FSH- to LH-dependency allows the dominant follicle to grow to preovulatory size and eventually reach ovulation. However, LH support is influenced by ovarian steroids progesterone and estradiol. In the absence of progesterone (i.e. during follicular phase) LH pulse frequency increases and dominant follicle grows more than 1 mm per day, releasing estradiol that acts in a positive

feedback with LH leading to the preovulatory LH surge and ovulation. On the other hand, under luteal levels of progesterone (i.e. during luteal phase), LH pulse frequency decreases and gonadotropin support of the dominant follicle is not enough to maintain preovulatory growth. As a reference, the largest preovulatory follicle requires about one pulse of LH every two hours to maintain its growth. The lack of this support leads to loss of steroidogenic ability and functional dominance of the dominant follicle, driving it to regression and finally atresia. FSH surge is released again and the emergence of a new follicular wave occurs.

Follicular dominance and the influence of gonadotropins and steroid hormones have transcendental weight in ovarian manipulation. Taken into account this information, most of the protocols have been modified during the last years in cattle, sheep and goats.

2.4. Corpus Luteum and Luteolysis

After puberty, the female acquires the ability to reproduce, showing sexual behavior and awakening the attractiveness to and from the male. Probably related to the optimization of the male sexual performance and the efficiency in the process of reproduction, the female becomes sexually receptive only during estrus, just before ovulation. Duration of the estrous cycle is in average 21 days in cattle (approximately 20 days in 2-wave cycles and 23 days in 3-wave cycles), 17 days in sheep (16 to 18 days) and 21 days in goats (19 to 22 days). After ovulation, the formation of the corpus luteum takes place and blood progesterone concentrations increases slowly until day 8-12, and these values are maintained during 3-7 days. If the female does not get pregnant, luteal regression occurs and progesterone concentrations fall drastically 12-14 days after ovulation in sheep and 15-18 days in cattle and goats. In pregnant females, progesterone secretion is a key factor for normal embryo development and maintenance of pregnancy. Insufficient progesterone secretion during the early luteal phase results in the development of compromised embryos with limited ability for survival until the maternal recognition period (14-16 days after insemination). Thus, an effective increase in progesterone is critical in stimulating proper embryo development. Secretion of adequate amounts of progesterone during luteal development requires precise luteinization of theca and granulosa cells to form luteal cells, neovascularization, and the establishment of a blood supply (angiogenesis). For that reason, synchronization protocols that induce ovulation should ensure an adequate periovulatory phase that will be crucial for normal development of the new corpus luteum and pregnancy maintenance.

Several hormonal interactions appear during the estrous cycle, involving gonadotropin releasing hormone (GnRH), gonadotropins (FSH and LH), ovarian hormones (estradiol, progesterone, inhibin), and prostaglandin (PG) $F_{2\alpha}$. Endocrine regulation of reproduction was further presented in Chapter 2. A precise comprehension of these interactions is absolutely required to understand the pharmacological management of the ovarian function.

The mechanism controlling the development, maintenance and secretion function of the corpus luteum involves mainly LH. Growth hormone, FSH and some others local regulatory compounds such as growth factors, ovarian peptides, steroids and

prostaglandins play different roles acting as luteotrophic factors. The preovulatory surge of LH induces ovulation and differentiation of follicular cells that form the corpus luteum. In addition, pulsatile release of LH is necessary for normal luteal development in cattle. However, pulses of LH did not appear necessary for luteal development in ewes or maintenance of progesterone secretion in cows or ewes. Luteinizing hormone release is controlled by progesterone by negative feedback exerted on GnRH. Early in the luteal phase, in absence of progesterone high LH pulse frequency is present supporting luteal development. While luteal phase progress and LH is less necessary for luteal maintenance, higher progesterone levels induce lower LH pulse frequency. Functional endocrine units of corpus luteum are represented by small and large luteal cells, which are derived from theca and granulosa cells of the follicle, respectively. Small cells provide approximately 15-20% and large cells more than 80% of blood progesterone levels.

Prostaglandin $F_{2\alpha}$ is the major responsible for luteolysis at the late luteal stage of the cycle. Its synthesis occurs in the endometrium and is controlled indirectly by estradiol and progesterone. It is proposed that, toward the end of the luteal phase, progesterone action decreases both centrally in the hypothalamus and in the uterus due to the downregulation of progesterone receptors by progesterone. This downregulation may allow the return of estrogen action blocked during luteal phase, acting both centrally in the hypothalamus and peripherally in the uterus. Estrogen present on day 12 to 15 of the cycle stimulates the hypothalamic oxytocin pulse generator and produces a series of intermittent episodes of oxytocin secretion. At the same time, in the uterus, the return of estrogen action upregulates endometrial oxytocin receptors. The interaction of oxytocin release with oxytocin receptors in the endometrium leads to the secretion of luteolytic pulses of uterine $PGF_{2\alpha}$. In ruminants, additional source of oxytocin is released by the corpus luteum. Luteal oxytocin in these species may thus serve to amplify neural oxytocin signals that are turned into pulses of $PGF_{2\alpha}$ by the uterus. Finally, the luteolytic mechanism results in progesterone decrease to basal levels in less than one day. For all that, $PGF_{2\alpha}$ has been widely used in the manipulation of estrous cycle in ruminants for several decades, representing together with progesterone the two main hormones used for ovarian control.

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Biographical Sketches

Alejo Menchaca, DVM, MSc, PhD, was born in Uruguay in 1973 and received his veterinary degree from Universidad de la República (UdelaR) in Montevideo in 2000. He received his MSc degree in Animal Production from Faculty of Veterinary program (UdelaR) in 2005 and his PhD degree in Biology (physiology) from PEDECIBA (Program for the Development of Basic Sciences, UdelaR) in 2009. His major field of study includes ovarian physiology, estrus synchronization, fixed-time artificial insemination, superovulation and embryo transfer, and *in vitro* embryo production in ruminants. He was enrolled as a Research Assistant at the Department of Physiology at UdelaR early in 1997 being a graduate student, and he remained for 10 years in the Laboratory of Physiology of Reproduction at this University. Dr. Menchaca founded Fundación IRAUy in 2009, a non-profit organization with focus on research and development in biology and animal reproduction. He is currently President of Fundación IRAUy and Director of Instituto de Reproducción Animal Uruguay, an institution focused on services and consulting in animal reproduction. He is Professor of the Postgraduate Programs of Facultad de Veterinaria and PEDECIBA, UdelaR, Uruguay. Additionally, he is also permanent consultant for pharmaceutical industry in the field of animal reproduction. He has numerous publications in peer review journals, international meetings and book chapters. He is frequently invited as speaker in scientific and technical meetings, as well as he acts as reviewer of about 20 international journals. Dr. Menchaca is 1st Level researcher of the National System of Researchers from National Agency for Research and Innovation (*Agencia Nacional de Investigacion e Innovacion*), Uruguay. He is member of the International Embryo Transfer Society (IETS), International Society for Transgenic Technologies (ISTT), and Society of Veterinary Medicine of Uruguay. He has been recipient of important awards in Uruguay from National Academy of Veterinary Sciences (2008) and the "Award Dr. Caldeyro-Barcia in Biology" for his scientific contribution in ovarian physiology (2011).

Gabriel Bó, DVM, MVSc, PhD, is currently President and Director of Research and Post-graduate training of the Instituto de Reproducción Animal Córdoba (IRAC) and Director of the Specialization and Masters programs on Bovine Reproduction at the National University of Cordoba. He was born in Argentina in 1962 and received his veterinary degree from the Facultad de Ciencias Veterinarias, Universidad Nacional de Rosario in 1985 and he received his M.V.Sc. (1991) and Ph.D. (1995) degrees in the Department of Herd Medicine and Theriogenology, Western College of Veterinary Medicine, University of Saskatchewan. Dr. Bó has been working on applied research and his main interests are follicular development, superovulation, recipient management, estrus synchronization, and embryo development and freezing. He is not only involved with research and teaching, as IRAC is a client-focused research and service institute (through the company Biogen Argentina SA) he practices embryo transfer and provides fixed-time artificial insemination services to producers in the Central and North part of Argentina. The programs developed under the supervision of Dr Bó, have been regarded as one of the major contributions to the application of fixed-time artificial insemination programs in Argentina, Brazil, Uruguay, Colombia and other countries. Dr. Bó is currently President of the International Embryo Transfer Society (IETS). He has also been President of the Argentine Chamber of Reproductive Biotechnology and Artificial Insemination (CABIA). In 2008 Dr. Bó obtained the "Taurus Award" in recognition of his scientific and academic contribution to the field of Bovine Reproduction. He has lectured in several short-courses on advanced reproductive technologies in several countries around the world. He has been an invited speaker and has participated in workshops at several meetings around the world, including the International Embryo Transfer Society, International Congress of Animal Reproduction (ICAR) and the World Buiatrics Congress.