Chapter 05

Hormonal Protocols in Small Ruminants

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Abstract

The reproduction in small ruminants can be controlled by the administration of exogenous hormones to modify the physiological chain of events involved in the estrous cycle, knowledge about the physiology of the follicular development in the ovary and, in particular, the relationships established among follicles developed over the same time in the ovaries and the interaction with the hormones that control reproduction is essential for an adequate use of assisted reproduction protocols. The manipulation of the estrous cycle can concentrate the insemination and births, as well as induce cyclicity, shorten the interval between births, schedule births for a favorable season of the year with available feed, and use genetically improved animals. The modifications involve the estrus synchronization by reducing or increasing the duration of the luteal phase, stimulating follicular development, and inducing ovulation. A multiple ovulation and embryo transfer (MOET) program are based on estrus induction or synchronization and superovulation of donor animals, followed by artificial insemination and collection of embryos with uterine lavage, and subsequent transfer to recipient females or cryopreservation, traditionally includes the insertion of a vaginal pessary with progesterone analogs such as fluorogestone acetate (FGA) or medroxyprogesterone acetate (MAP), or even progesterone itself, using a controlled internal drug releasing (CIDR) device for progesterone, which is used for 12 or 14 days in order to induce and/or synchronize the estrous cycle. Exogenous administration of gonadotropins begins two days before the removal of the vaginal pessary in order to stimulate follicular growth and provide multiple ovulations. Hormonal protocols for follicular aspiration consist of stimulating the follicle growth with ECG and/or FSH to obtained greater follicles available and greater diameter for aspiration, which can increase 2 to 4 follicles per session, resulting in a better response in the recovery, maturation, and in vitro production of embryos.

Introduction

The control of the estrous cycle with hormones allows the producer to use different breeding biotechniques to increase the productivity of the herd, such as the selection of breeding animals, acceleration of the process of genetic gain, or the choice of the best breeding season, thus facilitating management. The method of mating will also influence the choice of the best hormonal technique and protocols to be used.

The reproduction of small ruminants can be controlled by the administration of exogenous hormones to modify the physiological chain of events involved in the estrous cycle, which is related to the number of follicles present in the ovaries at the start of treatment with gonadotropin, their ability to grow to pre-ovulatory stages, and the elongation or shortening of the luteal phase.

Reproductive Physiology and Hormonal Interaction

Reproductive manipulation often depends on the control of the ovarian follicular development. Knowledge about the physiology of the follicular development in the ovary and, in particular, the relationships established among follicles developed over the same time in the ovaries and the interaction with the hormones that control reproduction is essential for an adequate use of assisted reproduction protocols.

The development of the ovarian antral follicles follows a wave like pattern. The follicular development of each wave is preceded by an increase in the serum concentrations of FSH, lasting 3 to 4 days. The interval of a follicular wave varies from 3 to 5 days, with three or four follicular waves during the complete estrous cycle. A follicular wave is determined by the synchronized growth of a group of follicles (emergence), followed by one or more follicles that continue to grow (dominant follicles), while other follicles regress (subordinate follicles). One to three ovarian antral follicles emerge or grow from a

group of small follicles (1 to 3 mm in diameter), reaching a diameter of ≥ 5 mm before regression or ovulation [1].

The concentration of serum estradiol increases together with follicle growth in each wave, with a peak around the end of the growth phase of the largest follicle. However, when compared to the other waves, the ovulatory wave is the one with the highest concentration of estradiol from an increase in the frequency of LH pulses during the end of the luteal phase, which stimulates the production of estrogen. Estradiol and inhibin regulate the secretion of FSH in small ruminants through negative feedback mechanisms. During the luteal phase, progesterone blocks the stimulation of the release of the preovulatory LH peak by the estradiol in the waves before the preovulatory one [2].

It is established that, during folicular phase, an increase in the estradiol level stimulates the neurosecretory system to increase GnRH secretion. Consequently, GnRH induces a LH surge, ovulation, and the subsequent luteal phase. In most species, the development and final maturation of antral follicles after luteolysis is dependent upon an increase in LH pulsatile secretion, as the concentration of FSH decreases because of the high concentration of estradiol and inhibin A secreted by the ovulatory follicle. It is known that LH is an essential requirement for normal ovarian follicle development and subsequent luteal function. The response to GnRH will depend on the endocrine environment before the induced increase in LH, which is more important than the size of the follicle to determine the ovarian response [1].

The concentration of progesterone is usually undetectable at the beginning of the estrous cycle and it then rises gradually between 2 to 8 days until reaching a maximum concentration of 1.5 to 3ng/mL as luteogenesis is complete. This concentration remains relatively constant between days 8 and 14 in sheep and up to 17 days in goats. After luteolysis, the concentration of progesterone drops back to undetectable levels. The use of exogenous progesterone is based on the principle that it acts modulating the release of gonadotropins by the pituitary.

The sudden increase in the concentration of progesterone reduces the release of LH, which causes atresia of the follicles dependent on this gonadotropin, and consequently reduces the concentration of estrogen produced by these follicles. In this way, new follicles are recruited starting a new wave.

The reproduction of small ruminants can be controlled by several methods developed in the last decades. Some of them involve the administration of hormones that modify the physiological chain of events during the estrous cycle. These modifications involve the estrus synchronization by reducing or increasing the duration of the luteal phase, stimulating follicular development, and inducing ovulation.

Hormonal Protocols for Estrus Synchronization/Induction

The manipulation of the estrous cycle can concentrate the insemination and births, as well as induce cyclicity, shorten the interval between births, schedule births for a favorable season of the year with available feed, and use genetically improved animals. Estrus synchronization/induction began to be studied in the 1960s with the use of synthetic progesterone analogs, which are used to change the physiological estrous cycle by manipulating the luteal or follicular phase.

In small ruminants, estrus synchronization is widely used but with varying results, with the reduction of the length of the luteal phase using prostaglandin F2α and its analogs or exogenous progesterone or progestogens. Progesterone acts increasing the luteal phase and its associations with hormones that promote follicular development and induce the ovulation.

The success of artificial insemination programs depends on a greater number of females in estrus that ovulate in synchrony in a short period, mainly in fixed-time artificial insemination (FTAI) programs. Therefore, for a good fertility rate after FTAI, the estrus synchronization treatment needs to provide a high degree of synchrony in relation to the time of onset of estrus/ovulation. Hormonal proto-

cols for estrus synchronization can also be used for the synchronization of recipient females for embryo transfer.

The most widely used protocol for estrous synchronization is based on long treatment, 12 to 14 day for sheep and 11 to 17 day for goats, with progestogen/progesterone (P4). The P4 is usually given as vaginal pessaries containing synthetic analogs (medroxyprogesterone acetate - MAP or fluorogestone acetate - FAP) or an intravaginal device impregnated with natural progesterone. Subcutaneous implants or intramuscular injections may also be used to simulate the action of endogenous progesterone produced by the corpus luteum after ovulation [3].

In the first protocols used, it was believed that the duration of the P4 treatment should be equal to or greater than the life of the corpus luteum (12 to 14 days) during a complete estrous cycle for an effective synchronization. The long use of P4 is currently known to induce subluteal levels of serum progesterone at the end of treatment, leading to an excessive period of follicle growth and oocyte aging. Shorter protocols using vaginal pessaries (5 to 7 days) have the advantage of maintaining the concentration of progesterone at appropriate luteal levels to stimulate follicular renewal and to induce ovulation of new follicles while achieving similar conception rates. Animals show estrus two to three days after suppression with P4 treatment [4,5].

Gonadotrophins are used together with P4, at the end of the protocol, in order to aid estrus synchronization and ovulation. The most commonly used gonadotropins are the Equine Chorionic Gonadotropin (ECG), which is essential for the induction of ovarian activity in small ruminants in anestrus, and the Follicle-Stimulating Hormone (FSH) [6]. The ECG doses range from 250 to 1000 IU, while the FSH doses range from 10 to 20 mg, depending on age, reproductive season, body condition, species, and breed. After treatment with P4 and gonadotropin, the animals tend to manifest estrus in approximately 48 hours and ovulation occurs in approximately 60 hours [7,8].

Prostaglandin F2α (PGF2α) can be used for estrus synchronization in two applications at different intervals or different associations

for short-acting protocols with P4, gonadotropins, and ovulation inducers, such as GnRH, in an attempt to increase the ovulation rate. Its effectiveness depends on the presence of a functional corpus luteum, and it should be applied between the 3rd and 13th day of the estrous cycle. When two applications are performed at an interval of 7 to 12 days, all animals are expected to present estrus. The interval between the administration of $PGF2_{\alpha}$ and the onset of estrus can vary, from 2 to 5 days, because of the phase of follicular development at the time of its application, and a greater variation can occur in relation to the time of onset of estrus, which limits the use of PGF2α for FTAI. Thus, protocols based on PGF2α should be used with the observation of estrus or together with an ovulation inducer [9].

The use of ovulation inducers in estrous synchronization protocols aims to induce and synchronize ovulation, in this way the animals tend to ovulate close to each other, which benefits the use of FTAI, since the success of the program will depend on the synchronization between the moment of insemination and ovulation. This may contribute to increased conception rates, especially with the use of frozen semen. The most commonly used hormones for this purpose are the synthetic Gonadotropin-Releasing Hormone (GnRH) analogs, such as buserelin, gonaderelin, and licerelin [5]. The GnRH analogs are used at the end of estrus synchronization protocols, between 12 and 36 hours after removing the vaginal pessary or 24 hours after the application of PGF2α [10]. Ovulation occurs approximately 27 hours after hormone administration. Estradiol benzoate may be an alternative to induce ovulation in small ruminants, but it is used on a smaller scale.
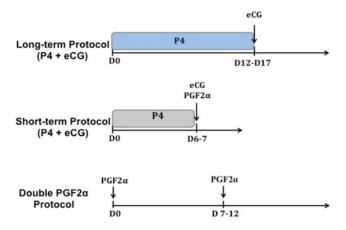
Figure 1: Hormonal protocols used for estrus synchronization in small ruminants.

Table 1: Pregnancy rate after hormonal protocols for artificial insemination.

Author	Protocol	AI	Semen	Pregnancy Rate
[4] Viñoles et al., 2001	MAP (12 days) + eCG	Natural matting	Fresh semen	67% B
	MAP (12 days)			63% B
	MAP (6 days) + eCG			58% B
	MAP (6 days)			87% A
[11] Oliveira-Muzante et al., 2011	PGF2α with a 7-day interval	Superficial cervical	Fresh semen	42%
[12] Oliveira-Muzante et al., 2013	PGF2α with a 7-day interval + GnRH 36 h after	Superficial cervical	Fresh semen	33.70%
[13] Vilarino et al., 2013	MAP (6 days) + eCG + PGF2α	Laparoscopy	Frozen semen	80.40%

Superovulation Protocols

A multiple ovulation and embryo transfer (MOET) program are based on estrus induction or synchronization and superovulation of donor animals, followed by artificial insemination and collection of embryos with uterine lavage, and subsequent transfer to recipient females or cryopreservation. The possibilities offered by the transfer of embryos, as a method of rapid multiplication of the number of offspring of a given female, are of great interest both from the basic point of view, in the research on embryonic development, and from the practical aspect of animal production. The increase in the number of offspring per female makes this technique an instrument of genetic progress, as it increases the selection pressure and also reduces the interval between generations. Among other applications, it facilitates commercial procedures for the import and export of genetic material under health guarantees and allows the preservation of animals at risk of extinction.
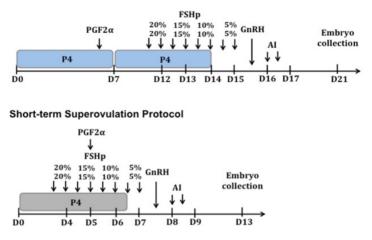
Despite advances in recent years, there is still variability in the in vivo production of small ruminant embryos, which is possibly related to the high variation in superovulatory response, which limits the diffusion of this technology. The superstimulatory treatment and the difference in the composition of commercially available follicle-stimulating hormone (FSH) preparations are seen as some of the causes [14]. The success of the ovarian response to superovulatory treatment also depends on factors such as follicular condition, genetics, season, and nutritional status of animals.

This variability in superovulatory response is the main limiting factor for MOET programs in small ruminants. Between 20 and 40% of the treated females do not respond to the superovulation treatment. The variability in the onset of estrus and the LH peak after hormonal treatment and the lack of synchrony at the onset of superovulation are among the problems, which can lead to failed fertilization [15].

Race is also a widely recognized variation factor in superovulatory response. The first studies with superovulation of small ruminants have established that most of the differences in superovulatory response were related to the prolificity of the different races used in MOET programs, and they have observed that highly proliferative races respond better to a stimulus with exogenous FSH.

In small ruminants, a multiple ovulation and embryo transfer (MOET) program traditionally includes the insertion of a vaginal pessary with progesterone analogs such as fluorogestone acetate (FGA) or medroxyprogesterone acetate (MAP), or even progesterone itself, using a controlled internal drug releasing (CIDR) device for progesterone, which is used for 12 or 14 days in order to induce and/or synchronize the estrous cycle [16]. Exogenous administration of gonadotropins begins two days before the removal of the vaginal pessary in order to stimulate follicular growth and provide multiple ovulations. Protocols using vaginal progesterone pessary for seven to eight days can achieve similar results [17] (Figure 2).

The most commonly used gonadotropins for superovulation of the donor are equine chorionic gonadotropin (eCG) and porcine (pFSH) or ovine follicle-stimulating hormone (oFSH). Of these, eCG was the first drug widely used for superovulation at a dose of 1000 to 2000 IU, one or two days before the removal of the vaginal pessary, in a single intramuscular injection. The eCG has a long in vivo half-life, thus it can result in a high incidence of anovulatory follicles, which are responsible for the high production of estradiol. The estrogenic condition caused by these follicles can likely change the transport of gametes through the genital tract and decrease the rates of recovery of embryos. The lower efficiency of eCG for in vivo production of embryos in the last three decades has contributed with the replacement of this hormone by porcine (pFSH) and, in a smaller scale, ovine commercial preparations (oFSH). Because of their short half-life, FSH preparations need to be administered repeatedly, ranging from six to eight applications, in decreasing doses, at 12-hour intervals, two to three days before removing the progestogen [18,19] (Figure 2).

Figure 2: Long- and short-term superovulation protocols commonly used in programs for the collection of embryos in small ruminants.

The ovarian follicular condition, at the beginning of the over-stimulation treatment, is of great importance for the final embryonic production. The ovulatory response and the total number of transferable embryos can be affected by the number of small follicles on the first day of the superstimulatory treatment and by the presence of a dominant follicle. For this reason, the desired superovulatory treatment begins during the emergence of the follicular wave or in the absence of established follicular dominance [20].

One approach that can be used to increase the control of the follicular growth is the "day 0 method", which consists of starting superstimulation with FSH on the day of ovulation, which coincides with the onset of the first follicular wave. In this protocol, sheep receive a short treatment with progesterone together with prostaglandin and eCG when the vaginal pessary is removed. Ovulation is induced, 36

hours later, with an application of a GnRH agonist. Superovulatory treatment begins 72-84 hours after the removal of the sponge, using 6 to 8 applications of FSH in decreasing doses, followed by the administration of PGF2α in the last two doses, while ovulation is synchronized with a single dose of buserelin (GnRH analog), administered 12 h after the last injection of FSH [21] (Figure 3).
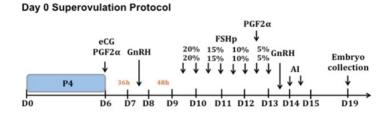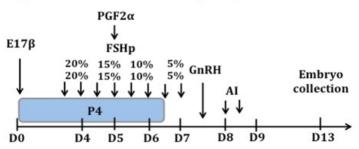
Day 0 Superovulation Protocol

Figure 3: Day 0 superovulation protocol, adapted from Menchaca et al. 2007.

Another alternative to synchronize the follicular wave emergence to begin the superovulation protocol is the combination of progesterone and estradiol. The mechanism responsible for suppressing the dominant follicle and inducing the emergence of a new follicular wave appears to be a systemic mechanism and involves both FSH and LH suppression for at least 24 hours. In sheep, the administration of 0.5 mg of 17β-estradiol or estradiol benzoate results in the emergence of a new follicular wave between 3.5 and 4 days later. Thus, the combination of progesterone and estradiol significantly reduces the variability of the follicular condition at the beginning of the overstimulation treatment (Figure 4).
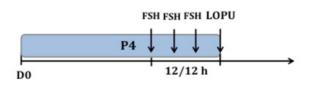
Superovulation Protocol using E17β

Figure 4: Superovulation protocol using 17β-estradiol for the synchronization of the follicular wave emergence at the beginning of the administration of FSH.

Repeat MOET programs can also influence embryonic production. Programs with sequential superovulation and collection of embryos, ranging from three to five procedures, can have a progressive decrease in the ovulation rate and, consequently, the production of embryos. The refractoriness of the ovary to successive hormonal treatments or adhesions caused by surgical procedures for the collection of embryos can have detrimental effects on the subsequent ovulation rate when superovulation is performed repeatedly. One practice that can be used for every two or three collection procedures is to allow the donor to go through a full gestation before starting the next MOET program.

Hormonal Protocols for Follicular Aspiration

Oocytes from small ruminants can be obtained with or without the use of hormonal treatments, and when hormones are used, follicular development is stimulated and thus follicles have greater availability and greater diameter for aspiration, which can increase 2 to 4 follicles per session, resulting in a better response in the recovery, maturation, and in vitro production of embryos.

Oocytes can be obtained in slaughterhouses, ovariectomy, and aspirated by laparotomy or laparoscopy (LOPU), the latter being more used because it is less invasive and can be repeated in a short time; this technique allows the use of animals that are genetically superior, pregnant, prepubescent, in seasonal anestrous, or those unfit for conventional reproduction.

Hormonal protocols for follicular aspiration consist of stimulating the follicle growth without ovulation, inducing follicular growth with FSH and eCG, and they may or may not use progestogen. The FSH or FSH:LH can be used in several doses or in a single dose and together or not with eCG. The eCG as a single injection is applied 48 hours before aspiration, and the association with FSH can be achieved with the application of 80 IU of FSH with 300 IU of eCG, administered 36 hours before follicular aspiration (Figure 5); thus, FSH can rapidly stimulate the initial follicular growth and eCG can allow the follicles to continue growing until aspiration.

LOPU Protocol using multiple FSH doses

FSH FSH FSH LOPU

P4

D0   12/12 h

LOPU Protocol using single eCG+FSH dose
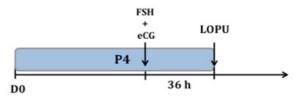
FSH
+
eCG   LOPU

P4

D0   36 h

Figure 5: Hormonal protocols for follicular aspiration.

Conclusion

Hormonal protocols can be used for estrus synchronization, superovulation, and collection of embryos, as well as follicular aspiration in order to reach the maximum reproductive potential of goats and sheep. However, the use of these hormonal protocols together with breeding biotechniques should consider the physiological, productive, economic, and feasibility aspects in order to achieve results that can effectively contribute to the genetic improvement and conservation of gametes.

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