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Reproduction Biotechnology in Farm Animals

Edited by
Tácia Gomes Bergstein-Galan

Preface

Animal production is based on tripod sanity, nutrition and genetic enhancement. However, without reproduction there is no animal production. Reproductive biotechnologies, when combined with rational food and sanity management, are substantial tools for accelerated genetic improvement and definitive genetic gain.

The book “Reproduction Biotechnology in Farm Animals” is intended to provide basic and applied information on the reproductive biotechnologies most used in farm animals to academics of veterinary medicine, professionals and rural producers. The authors of this work are renowned researchers of Brazil, with the differential beyond the theoretical basis also have profound practical knowledge of the topics covered.

In chapters 1 and 2, anatomo-physiological revisions in the male and female reproductive apparatus are contained of equines and bovine animals, respectively. Chapters 3 to 5 are focused on methods of manipulating the cycle of bovine, equine and small ruminants. The main reproductive biotechnologies applied in cows, sheep and goats are described in Chapters 6 and 7, while in Chapter 8 the promising embryos in vitro production is described in pigs. Chapter 9 is focused on andrology and reproductive biotechnology applied to the stallion. Chapter 10 covers physiology, semen preservation and artificial insemination of buffaloes.

Because it is a broad subject and in the process of accelerated evolution this book does not aim to exhaust all the important information of the reproductive biotechnics, but it provides theoretical basis for the main methods used in production species.

I thank all the authors who, so cordially, have agreed to join me in the elaboration of this book. For me, as editor, it was delightful to learn from great friends, colleagues and researchers. I wish you, dear reader, the same feeling.

Tácia Gomes Bergstein-Galan

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Chapter 1

Reproductive Physiology of the Equine

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Abstract

This chapter highlights the main aspects of reproductive physiology in the equine (mare and stallion). Puberty in mare usually occurs between 12 and 18 months, with sexual maturity being reached later. In the stallion, puberty occurs between 14 and 24 months of age, and reproductive maturity is only reached at 4 to 5 years. Reproduction in the equine species is strongly influenced by the photoperiod, and the reproductive season occurs in the months of long days (spring and summer). Between the reproductive season, or cyclic phase, and the anestrous or acyclic phase, there are two transitional periods (spring and autumn transition), which are marked by important peculiarities in the physiology of the mare. Control of folliculogenesis in a mare, spermatogenesis in stallion and steroidogenesis in both are performed by the hypothalamic-pituitary-gonad axis. The understanding of the functioning of this axis and the feedback relations that regulate it are of extreme importance for the understanding of reproduction in all species.

Puberty and Sexual Maturity

Puberty in females is marked by the first estrus or the first ovulation, but it is not synonymous of sexual maturity since it does not always represent the moment in which the female becomes capable of carrying a full term gestation. In fillies, puberty usually occurs between 12 and 18 months of age (on average 15 months), however, it var-

ies substantially (from 7.8 to over 27 months), and can be influenced by several factors such as birth season, body weight, breed, and lineage. Although they are capable of breeding, peripubertal fillies have high rates of the embryo or fetal loss and premature births when compared to mares mature sexually. Reproductive senescence is quite rare and most mares show cyclicity even at advanced ages [1,2].

In males, puberty can be defined as the moment at which the animal has the ability to reproduce successfully. Some researchers use the definition of puberty as the first moment in which the ejaculate contains 50 million spermatozoa with at least 10% motility. In foals, up to 8 months of age, the concentrations of gonadotrophins are low and the gonadal activity is minimal (infant period). The changes in the hypothalamic-pituitary-gonads axis mark the beginning of the pre-pubertal period when there is an increase in the secretion of LH and FSH between 8 and 10 months of age. Blood testosterone shows a large increase between 18 and 20 months of age, with puberty being reaching between the 20th and 21st months. However, the age at puberty varies from individual to individual, and may be precocious (14 months) or late (over 24 months), as it is influenced by factors such as birth, breed, breeding system and individual characteristics (as anatomical abnormalities). As in mare, puberty is not synonymous with sexual maturity, since a peripubertal animal does not have complete reproductive efficiency. A stallion is only

considered sexually mature when it reaches maximum reproductive capacity (testicular size and sperm production at maximum values), a phenomenon later than puberty, occurring on average up to 4 to 5 years of age [3–6].

Seasonality: Influence of Photoperiod on Equine Reproduction

Seasonal reproduction is a characteristic developed by some species, ensuring births concentrated in favorable seasons of the year. Equines exhibit a circannual reproductive rhythm, which is heavily influenced by changes in the photoperiod so that the mating season occurs in the months of long days (spring and summer). In the months of negative photoperiod (autumn and winter) the vast majority of mares do not have ovulatory cycles and stallions have lower fertility. There is a reduction in size and testicular activity, with a consequent decrease in the release of testosterone, sperm production and libido [5,7–9].

The pineal gland is the organ responsible for the production of melatonin in mammals. The luminosity, detected by the retina, controls its rhythmic secretion, which occurs at high levels during the night and low during the day. Thus, the photoperiod exerts influence on the secretory pattern of melatonin, a hormone responsible for regulating the hypothalamic production of gonadotrophins (GnRH) [10]. In equines, during the months of negative photoperiod (short days) the increase in melatonin lev-

els leads to a reduction in GnRH secretion. In addition to environmental factors, such as photoperiod and ambient temperature, age, nutrition, and body condition also exert influences on reproductive activity [1,8].

Reproductive Endocrine Control

Hypothalamic-Pituitary-Gonad Axis (HPG)

Understanding the functioning of the hypothalamic-pituitary-gonadal axis (HPG), including the participating hormones and feedback relationships between them, represents the basis for the study of reproductive physiology. The organs and hormones involved in this axis are responsible for correct steroidogenesis and spermatogenesis in the stallion, as well as for the ovulatory estrous cycles in the mares. The organs that make up this axis are the hypothalamus, located in the encephalic base, the pituitary, divided into adenohypophysis and neurohypophysis, and the gonads (testicles or ovaries) [11]. The major hormones involved in the HPG axis include gonadotrophin-releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estrogen, inhibin, and progesterone [12].

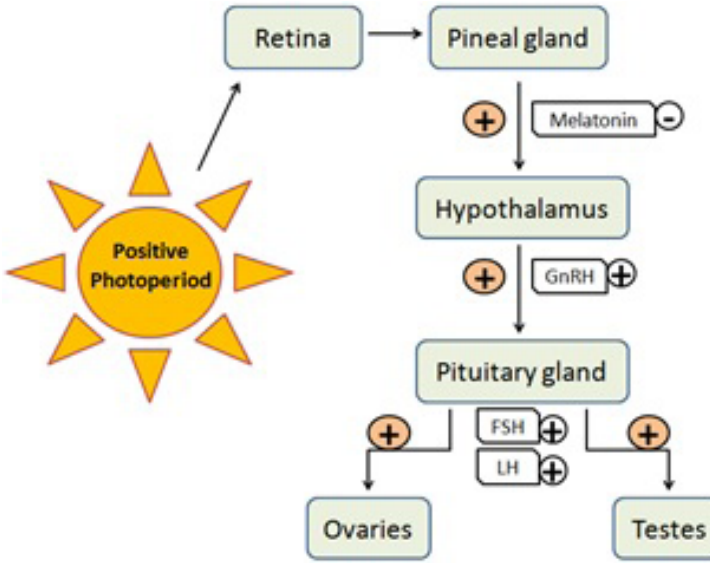

Figure 1: Influence of the photoperiod on the hypothalamic-pituitary-gonad axis in horses.

Table 1: Main organs or structures and hormones produced involved in reproductive control in horses.

Organ/Structure	Hormone
Pineal gland	Melatonin
Hypothalamus	Gonadotropin-Releasing Hormone (GnRH)
Pituitary gland	Follicle-Stimulating Hormone (FSH) and Luteinizing Hormon (LH)
Ovaries (Folicles/CL)	Estrogen and Inibin / Progesterone (P4)
Uterus	Prostaglandin F2α (PGF2α)
Endometrial cups	Equine Chorionic Gonadotropin (eCG)
Testes	Testosterone and Estrogens

Endocrine Control of Estrous Cycle and Initial Gestation in Mare

As described above, equine reproductive activity is strongly influenced by environmental factors, such as photoperiod, nutritional status, energy balance and stress [13]. The main stimulus for the initiation of cyclicity in the mare is the increase in the hours of daily luminosity. In this species, the decrease of melatonin secretion by the pineal gland (positive photoperiod) represents a stimulus for the hypothalamic release of GnRH, which is suppressed in the short-day months [1,8,10]. GnRH is responsible for regulating the synthesis and secretion of pituitary gonadotrophins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) [13]. These two hormones are produced by gonadotrophs (cells present in the pars distalis and pars tuberalis of the pituitary gland) and both exert their effects on the gonads [14].

FSH, as its name implies, is responsible for stimulating follicular growth. This growth occurs in the form of follicular waves, which are detected during transrectal ultrasonography by the simultaneous development of multiple follicles [15]. The mares may present one or two follicular waves per estrous cycle, being more common the occurrence of a single wave [16]. The waves can be classified as major and minor. Major waves are those in which follicular divergence occurs, with the formation of dominant and subordinate follicles. In the minor waves there is

no divergence and, consequently, there is no formation of dominant follicles or ovulation. Major waves can be primary or secondary waves. The primaries emerge during the diestrus and result in primary or estrous ovulations. The secondary ones emerge during early estrus or diestrus and give rise to anovulatory follicles or result in ovulation during diestrus [17]. Doubling ovulations can occur naturally in mares. The dominant follicles may be either from a single primary wave or synchronous ovulation may occur from a dominant follicle of the primary wave and another from the secondary wave [15].

During the 6 days preceding a primary wave, FSH is secreted in increasing concentrations, which remains elevated until emergence. In minor waves, this increasing secretion occurs for a smaller interval, in average 4 days. One to two days after the onset of the dominant follicle of the primary wave, FSH levels decrease [15]. The follicles under development produce two hormones, estradiol, and inhibin, which give negative feedback on the release of FSH [18]. During this phase of divergence, intrafollicular factors such as estradiol, inhibin-A, IGF1(insulin-like growth factor 1) and vascular endothelial growth factor (VEGF) act on the dominant follicle, sensitizing its cells. The sensitized follicle is uniquely capable of maintaining its development under low levels of FSH. Subordinate follicles undergo atresia [19].

Estradiol, produced mainly by the dominant follicle, presents increasing levels during estrus, reaching the peak

two days before ovulation [18]. This hormone is responsible for behavioral changes (raised tail, rhythmic clitoris exposure, frequent and passive urination, abduction of the pelvic limbs, "squatting", vocalization), changes in the reproductive tract (uterine edema, cervix rosea, moist, relaxed and open cervix) and sexual receptivity of the mare during the characteristic estrus[20,21]. In addition, estradiol exerts positive feedback on LH release when circulating concentrations of progesterone are low [22]. Thus, the pre-ovulatory period is marked by a drop in FSH levels and an increase in LH, which acts on the final growth and maturation of the dominant follicle, culminating in ovulation [23]. The dominant follicle reaches about 40 mm in the preovulatory period [1].

Ovulation consists of rupture of the dominant follicle in the ovulation fossa, resulting in the release of oocyte and cumulus cells, granulosa cells, and follicular fluid. The cells, of the granulosa remaining at the ovulation site, undergo a process of luteinization by the action of LH and begin to compose the primary CL. Luteinization modifies the secretory activity of granulosa cells, which cease to secrete estradiol and secrete progesterone (P4) [24]. The estrus phase of the mare where there is an active CL is called the luteal or diestrus phase and is marked by the action of P4 on the reproductive tract (absence of uterine edema, pale, dry and tightly closed cervix) and behavior, extinguishing the sexual [21].

P4 realizes negative feedback on the release of LH, causing the levels of this hormone to remain low during

the diestrus. In mares, secretion of P4 begins on the day of ovulation (D0), and blood concentration reaches maximum values after 6 days (D6) [25,26]. When there is no gestation subsequent to ovulation, the uterus produces prostaglandin F2α (PGF), a potent luteolytic substance. The PGF reaches the ovary via systemic circulation and triggers the lysis of CL around the D14 of the estrous cycle [26–29]. After luteolysis, P4 levels drop, allowing the onset of a new follicular phase.

When oocyte fertilization occurs in the oviduct ampulla, there is the formation of an embryo that enters the uterine horn, in the initial morula or blastocyst stage, between D5 and D6[30,31]. Once in the lumen, the embryonic intrauterine mobility phase is initiated, an essential phenomenon for the maternal recognition of pregnancy to occur. The equine embryo is coated with a capsule which, in addition to presenting anti-adhesive properties, maintains its turgidity and spherical shape, preventing its early fixation and favoring mobility promoted by uterine contractions [27]. The presence of the embryo in contact with the endometrium, migrating throughout the uterine lumen, is necessary to block the release of PGF and, consequently, luteolysis. This blockade needs to be effective for the primary CL to remain functional and to remain secreting P4, the hormone responsible for the maintenance of gestation [32]. Between days 15 and 17, mobility is terminated, with embryonic fixation occurring, usually at the bifurcation of one of the uterine horns [33,34].

Between days 28 and 35 of gestation, the formation of endometrial cups occurs, consisting of mixed structures of

placental origin (related to conception) and maternal (related to the uterus) [25]. The endometrial cups are responsible for the production of equine chorionic gonadotropin (eCG), a hormone present in the blood of mares from 40 days of pregnancy, reaching maximum levels at 80 days and disappearing at 150 days [35]. The eCG is responsible for the resurgence of primary CL and the development of multiple secondary CLs in the maternal ovaries, which remain functional until 150 days of gestation [35,36]. These CLs (primary and secondary) are responsible for the transient increase and prolongation of P4 secretion, which is necessary until placental production is sufficient to maintain pregnancy until delivery [27].

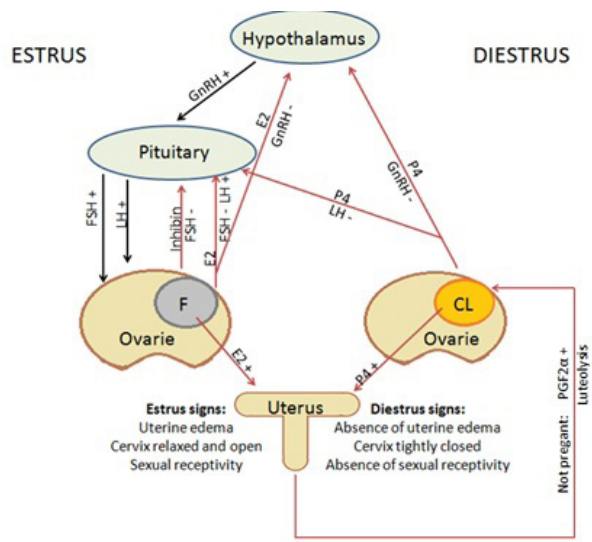

Figure 2: Hypothalamic-pituitary-ovary axis and their interactions with the uterus in the non-pregnant mare.

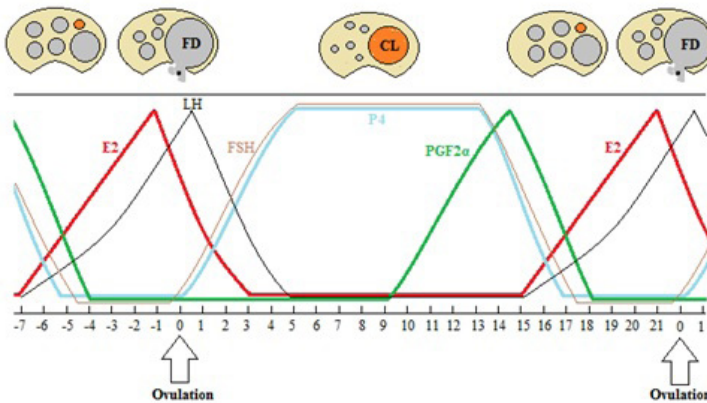

Figure 3: Hormonal variations during the estrus cycle of the mare. FSH - follicle stimulating hormone; LH - luteinizing hormone; E2-estradiol; P4 - progesterone; PGF2α - prostaglandin F2α; FD - dominant follicle; CL - corpus luteum.

Transitional Periods

Since mares are classified as long-day seasonal polyestrus, they are known to have two well-defined reproductive seasons throughout the year, according to the photoperiod. The cyclic or fertile season, in which there is follicular development followed by ovulation and formation of functional CL, occurs from mid-spring, extending through the summer. The anestrous season, or ovarian quiescence, predominates during late fall and all winter. Between the cyclic and anestrous phases, there are two periods that merit attention, termed spring transition and autumn transition. The spring transition occurs between the end of the anestrous season and the beginning cyclic season since the autumn transition occurs at the end of the

breeding season and beginning of the anovulatory phase. Both these periods are marked by important peculiarities.

During the anovulatory season, there are mares that present follicular development accompanied by characteristic estrus; however, ovulation does not occur, leading to low pregnancy rates during winter and early spring [25]. At this negative photoperiod, there is a reduction in GnRH secretion by the hypothalamus, as well as in LH synthesis. When the days become longer, increased secretion of GnRH stimulates the release of FSH, however, the release of LH is only stimulated later, due to the lack of pituitary reserves. Since FSH secretion is stimulated during the spring transition, follicular development is initiated, however, the follicles do not produce significant amounts of estradiol [37]. Thus, during the transition period of spring, long anovulatory estrus occurs due to follicular growth followed by atresia. This is caused by insufficient levels of LH to induce ovulation [23]. Several waves of follicular growth and atresia occur during this phase until a competent follicle develops. This follicle is able to secrete sufficient amounts of estradiol to induce the synthesis and release of LH by the pituitary, leading to the first ovulation of the year [37].

In some horse breeds the birth of foals at the beginning of the equestrian year (which begins on January 1 in the northern hemisphere and July 1 in the southern hemisphere) is prioritized. For this to be possible, mares need

to become pregnant in periods unfavorable to cyclicity (mid-February in the northern hemisphere and mid-August in the southern hemisphere). Thus, several strategies are employed to anticipate follicular development in these matrices. The use of light therapy, associated or not with the use of hormone therapy, is the most used method [38]. It has also been shown that exposure to a stallion during the transition period is able to anticipate the reproductive season in females. Therefore, keeping mares in proximity to one or more stallions may represent an alternative or complementary strategy to hormone therapies and light programs [39].

The autumn transition is marked by a commitment of the luteal function, with consequent reduction of progesterone secretion. It is also common during this period the occurrence of failures in the luteolytic mechanism, resulting in prolonged diestrus [40,41]. In addition to changes in luteal function, follicular anovulatory waves occur frequently during the autumn transition, which precede complete anestrous. The duration of the transition period, as well as the time of entry into anestrous, varies individually. The time of entry into anestrous does not influence the return to cyclicity in the subsequent breeding season. Thus, the duration of anestrous varies considerably between mares [42].

Endocrine Control of Stallion Reproduction

As previously seen, the equine species shows a seasonal reproduction. The influence of the season on reproduction in the stallion is not as marked as in the mare, since stallions maintain reproductive activity throughout the year, with a reduction of fertility during the months of negative photoperiod. During the spring and summer months, there is an increase in the testicular function, as well as the concentrations of FSH, LH, and testosterone in the serum, due to the increase in hypothalamic GnRH secretion (in response to the low production of melatonin by the pineal). Consequently, during these seasons, sexual behavior also becomes more exacerbated [5,7,9].

As in mare, GnRH, produced by the hypothalamus, is secreted in the pituitary portal system and exerts its function directly on the pituitary, controlling the synthesis and secretion of LH and FSH. In the stallion, LH has a steroidogenic function, stimulating the production of androgens (testosterone and dihydrotestosterone) and estrogens by the Leydig cells present in the testicles [11,43]. FSH acts on Sertoli cells (also present in the testicles) stimulating the production of substances necessary for spermatogenesis, such as estrogens, inhibin, and activin. Testosterone realizes negative feedback on the hypothalamic release of GnRH. The inhibin and activin act on the pituitary gland, activin appears to stimulate the synthesis

and secretion of FSH, and inhibin makes negative feedback on it [11,12,44].

FSH is essential for the onset of spermatogenesis during puberty [45]. Testosterone has intratesticular and peripheral effects. This hormone acts stimulating sperm production and promoting a normal epididymal function, is essential for the maintenance and restoration of spermatogenesis in adults. In addition, it is responsible for the male characteristics, for the libido, and for properly maintaining the functions of the accessory sexual glands [11,46].

Testicular Descent

The displacement of the testicles from the abdominal cavity towards the interior of the scrotum is called the testicular descent. The anatomical structures involved in the testicular descent include the inguinal canal, the vaginal process, and the gubernacular ligament. The inguinal canal consists of the passage between the inner inguinal ring (in contact with the abdominal cavity) and the external inguinal ring (which gives access to the interior of the scrotum). The vaginal process consists of an evagination of the peritoneum that passes through the inguinal canal into the scrotum. The gubernacular ligament connects the vaginal process to the scrotum and helps to keep the passage open [47]. The process of descent occurs in three stages: the nephritic displacement, the trans-abdominal displacement, and the inguinal passage. The first stage is completed at 60 days in the equine fetus, the second stage

ends with the testicles inside the inguinal canal, at 9 to 10 months of fetal life, and the end of the third stage represents the passage through the inguinal ring, completing the testicular descent in the last month of gestation or up to 10 days after birth. During the first two weeks after birth, the internal inguinal ring contracts and undergoes fibrosis, measuring approximately 1 cm in diameter, which prevents testicular movement in any direction [48,49].

The large size of the equine testicles makes the descent process difficult. Cryptorchidism, relatively common in horses, occurs when there are failures in testicular descent, and one or both testicles are not found in the scrotum. Retention of the testicle within the abdominal cavity (true cryptorchidism) occurs in cases where the testicle does not enter the inguinal canal before the closure of the inner inguinal ring. Since the left testicle is slowed in relation to the right, cases of left unilateral cryptorchidism are more frequent [49,50]. As the normal testicular function is injured at elevated temperatures, cryptorchid testicles are unable to produce normal spermatozoa, however, they are able to produce androgens. Thus, cryptorchid animals bilaterally are sterile but may exhibit stallion sexual behavior. Cryptorchidic testicles are more likely to present neoplasm and torsion of the spermatic cord. Since cryptorchidism is possibly genetic, stallions that exhibit this condition should be removed from reproduction. Some foals may present the testicles in the inguinal region at birth and, although it is considered abnormal, the descent into the scrotum may take weeks to months, usually occurring up to 2 to 3 years of age [51].

Spermatogenesis

Spermatogenesis begins near puberty and consists of the process of spermatozoa production from primordial germ cells, called spermatogonia, present in the germinal epithelium of the seminiferous tubules of the testicles [52]. This process can be divided into three phases. The first phase, called spermatocytogenesis, is related to the mitotic multiplication of spermatogonia, which occurs for the production of primary spermatocytes and new spermatogonia (maintenance of the germinative population). The second stage, called meiosis stage, involves meiotic divisions of the primary and secondary spermatocytes for the production of spermatids (haploids). The third stage, or spermiogenesis, is a stage of differentiation in which the spermatids undergo a set of cellular transformations that result in spermatozoa formation [52,53]. The major events of spermiogenesis include acrosome formation, nuclear condensation and stretching, cytoplasmic contraction, and flagellum formation. The release of newly formed spermatozoa into the lumen of the seminiferous tubules is a process called spermiation [51].

The occurrence of the three phases of spermatogenesis (from spermatogonia to spermiation) in a given region of the seminiferous epithelium is considered a spermatogenic cycle. In stallions, the duration of each cycle is 12.2 days, and the complete spermatogenesis takes approximately 4.5 cycles or 57 days. Thus, an interval of up to two months may occur between a deleterious event for

spermatogenesis and consequent drop in sperm quality. Likewise, a similar or greater interval will be required for reinstatement of normospermia following injury. Since the production of new spermatogonia is not synchronized between the tubules, nor simultaneously along the same tubule, at all times there are spermatogonia beginning the process of spermatogenesis and spermatozoa being spermied. In this way, the male is able to maintain the constant availability of gametes [52,54]. The daily sperm production in stallions is about $5x10^9$ spermatozoa (in both testicles), or $16x10^6$ spermatozoa per gram of testicular parenchyma [6].

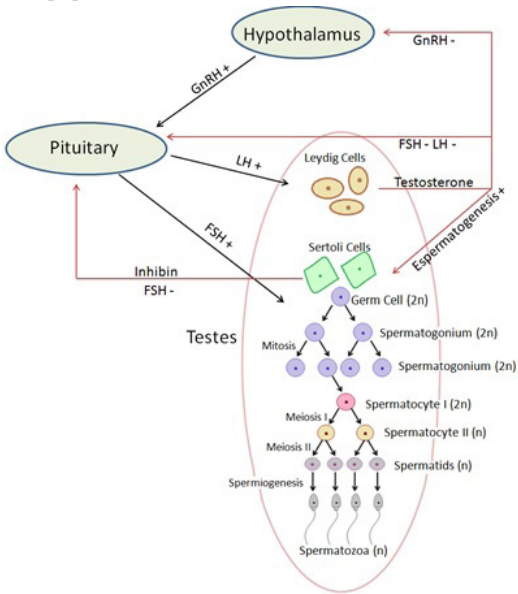

Figure 4: Hypothalamic-pituitary-testes axis, steroidogenesis, and spermatogenesis in the stallion.

Hemo-Testicular Barrier

The epithelium of the seminiferous tubules is composed of two major cell types, germ cells, and Sertoli cells. Sertoli cells extend from the base to the lumen of the seminiferous epithelium, are polarized and provide structural and nutritional support to germ cells. Through specialized cell junctions, Sertoli cells maintain contact between themselves and germ cells. This intimate intercellular contact is responsible for the formation of the hemo-testicular barrier, one of the tightest barriers in mammals. It separates the seminiferous epithelium into two compartments, called basal and adluminal [55]. The hemo-testicular barrier is fundamental for spermatogenesis, it is highly selective and determines which molecules may or may not enter the adluminal compartment. In this way, the hemo-testicular barrier is responsible for creating and maintaining a suitable microenvironment for the development of germ cells. It also has an immunological barrier function, segregating the systemic circulation of post-meiotic germ cell antigens [56].

Maturation, Transport, and Storage

Spermatozoa, produced by the germinal epithelium and released into the lumen of the seminiferous tubules, do not have fertilizing capacity *in vivo*. They need to undergo a post-testicular maturation process to acquire this function. The newly spermatozoa are carried by the seminiferous tubules and into the rete testis. From the

rete testis, the spermatozoa follow the efferent ducts to the head of the epididymis, where the ducts merge, forming a single epidydimal duct (one referring to each testicle). The epidydimal duct is approximately 45 meters long and follows a tortuous path through the head, body and tail of the epididymis, ending in the deferent duct. The initial segment of the head is responsible for the reabsorption of fluids and solids. The rest of the head and the body of the epididymis act on sperm maturation [50,57].

Among the sperm modifications due to epidydimal maturation, the most important are the acquisition of progressive motility (flagellar movement), the ability to recognize and attach to the zona pellucida oocyte, and the migration and release of the cytoplasmic drop. The cytoplasmic drop is composed of a remnant cytoplasm associated with the newly produced spermatozoon, during transit through the epididymis the drop moves from the base of the head towards the end of the flagellum, being released in the tail of the epididymis, during ejaculation or after ejaculation [58]. The tail of the epididymis and the deferent duct (proximal portion) are as reservoirs of fertile spermatozoa [50,57].

The spermatic transit of the head to the tail of the epididymis lasts about 4 days in the stallion, not being altered by the frequency of ejaculations. Transit through the head and body is constant and mediated by peristaltic contractions of smooth muscle, present in the duct's wall. The portion of the epidydimal duct that forms the tail remains quiescent until there is a stimulus for smooth mus-

cle contraction (eg, during ejaculation) [6,50]. In this way, sperm transit through the tail of the epididymis is influenced by the interval between ejaculations, being on average 7 to 8 days inactive stallions and 10 days in stallions in sexual rest. Inactive stallions, the number of spermatozoa present in the tail of the epididymis is about 20 to 30% lower than that of stallions in sexual rest, in which $54x10^9$ spermatozoa (on both sides) remain on average [50].

Thermoregulation

For spermatogenesis to occur properly, and the sperm stored in the epididymis maintain their fertilizing capacity, it is necessary that the testicular and epididymal temperature is below body temperature (3 to 5° C below). Important thermoregulation mechanisms work together to maintain proper temperature. These mechanisms include the action of the scrotum, dartos fascia, cremaster muscle, and pampiniform plexus. When the room temperature has elevated the musculature of the dartos fascia and the cremaster muscle relax, consequently, the scrotum becomes more pendular and away from the abdominal wall. This increases scrotal evaporation, maximizing testicular heat loss. In cold environments, the opposite occurs (contraction of dartos fascia muscles and cremaster muscle), the testicle is closer to the abdominal wall, reducing the scrotal surface and minimizing the loss of heat [11,20,50,59].

The pampiniform plexus, present in the spermatic cord, consists of a counter-current heat exchange system

between the artery and the testicular vein. The testicular artery carries arterial blood (body temperature) toward the testicles, and the testicular vein is responsible for the return of venous blood (testicular temperature) from the testicles to the body. The testicular artery divides into fine capillaries, which form a network, before entry into the testicles. The same happens with the testicular vein, which runs in the opposite direction to the artery. The capillary networks, of arterial and venous origin, maintain close contact, forming the pampiniform plexus. Since venous blood (testicular origin) is colder than arterial blood, this proximity between the capillaries allows a heat exchange, resulting in venous blood heating and arterial blood cooling (before entry into the testicles) [20,60].

Accessory Sex Glands

The accessory sex glands are responsible for the secretion of seminal plasma, which added to the spermatozoa compose the ejaculated semen. Seminal plasma consists of the largest fraction of the ejaculate. Among its functions are the transport of spermatozoa to the reproductive tract of the mare, stimulation of sperm motility (spermatozoa are immobile until mixed with seminal plasma), energy supply (usually in the form of glucose) for spermatozoa, oxidation, and maintenance of osmotic pressure [61]. The glands present in the stallion are the ampules of the deferent ducts, the seminal vesicles, the prostate and the bulbourethral glands. The ampules are located in the

end portion of each deferent ducts(urethral end) and are well developed in this species [62]. The secretion of the ampules comprises the pre-sperm fraction of the ejaculate and contains an antioxidant agent, ergothioneine[20].

The seminal vesicles are bag-shaped lobulated glands located on both sides of the bladder. Its secretion occurs directly in the urethra, through its own duct, separated from the deferent ducts[62]. These glands are responsible for a large part of the seminal plasma, composing the sperm-rich fraction and the gelatinous post-sperm fraction [20,61]. The prostate is caudally to the seminal vesicles, close to the junction of the bladder with the urethra [62], is bilobed and has a single outlet in the urethra. Prostatic secretion contributes to the pre-sperm fraction of the ejaculate. The bulbourethral glands are close to the root of the penis on each side of the urethra. Its secretion composes the pre-sperm fraction and the sperm-rich fraction [20,63].

Ejaculate Characteristics

The equine ejaculate can normally vary in volume between less than 40 and more than 150 mL, being constituted by three fractions. The first fraction (pre-sperm) has a watery appearance and scarce spermatozoa, the second fraction is rich in spermatozoa, and the third (post-spermatic) has a gel characteristic [64]. The normal coloration is off-white, with a general aqueous aspect [65]. The sperm motility of good quality semen should be at least 60% to

70% with a vigor between 4 and 5 (minimum 3). The average concentration varies between $50x10^6$ and $150x10^6$ spermatozoa per mL, with the total number of spermatozoa being ejaculated between $3x10^9$ and $9x10^9$[64,66]. When two successive collections are performed, with an interval of approximately one hour between them, the total number of spermatozoa in the second ejaculate is expected to be about half of the first. In the second collection, a normal stallion should be able to ejaculate at least $1x10^9$ sperm morphologically normal and with progressive motility [65]. In relation to the morphological examination of semen, it is considered that a breeder must present at least 70% of normal spermatozoa [66].

The characteristics of the ejaculate, especially with as regards to the spermatic concentration and the total number of spermatozoa in the ejaculate, are influenced by factors such as season, age and race of the stallion. During the months of positive photoperiod (physiological reproductive season) the spermatogenesis is stimulated, resulting in a greater daily sperm production. This increase in sperm production is due to the increase in Sertoli cell population, secretion of FSH, LH, and testosterone, with a consequent increase in testicular weight [67]. The age of the stallion also exerts an influence on semen quality, very young stallions (less than 3 years old) or older (more than 11 years old) presenting less voluminous ejaculates, with lower sperm concentration, lower total number of spermatozoa and a higher proportion of sperm abnormalities. Most ejaculate characteristics vary significantly among

different breeds. Ponies usually have a smaller seminal volume (about 20mL) with a total number of spermatozoa slightly above $1x10^9$ per ejaculate, whereas Arabian stallions have larger seminal volumes (average close to 40 mL), and can reach more than $12x10^9$ total spermatozoa per ejaculate [68]. When evaluating a stallion we must remember all the factors that exert influence on the seminal quality, in this way the results will be correctly and individually interpreted.

Conclusion

The study of physiology represents the basis for the understanding of reproduction in all species. It is important to remember that the activity of the hypothalamic-pituitary-gonadal axis in horses is strongly influenced by the photoperiod, leading to a physiological reproductive season during the months of long days. The knowledge of the hormones involved in the control of reproduction, and its mechanisms of regulation, are essential for the understanding of reproductive pathologies, as well as for the manipulation of the estrous cycle of the mares and the application of different reproductive biotechnologies.

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Chapter 2

Bovine Reproductive Physiology and Endocrinology

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Abstract

This chapter is about the reproductive physiology of females and males of the bovine species, and the main differences between *Bos taurus taurus* and *Bos taurus indicus* animals. The reproductive function of cattle is regulated by hormones which interact through the hypothalamus-pituitary-gonadal axis, being influenced by nutritional, sanitary and management factors. From sexual maturity, females and males become fit for reproduction, and are able to breed all year long, suffering little or no influence from the brightness of days.

Reproductive Physiology of Bovine Female

Puberty and Sexual Maturity in the Cow

Females puberty is characterized by the first oestrus with ovulation [1] and in *Bos taurus indicus* heifers puberty is later than *Bos taurus taurus* heifers [2]. Female bovines of European breeds reach puberty between 9 and 12 months of age, and those of beef cattle breeds at around 24 months [3]. Thus, puberty refers only to the early reproductive life of the female, but for the introduction of heifers in reproduction, it is important that they have reached sexual maturity regarding body development in order to avoid possible complications during childbirth [4-6].

Environmental and nutritional factors associated with postweaning weight gain are important factors that reduce the age at puberty [7]. Heavier heifers reach puberty at younger ages [8]. The development of the reproductive tract is a useful tool for the evaluation of puberty in heifers [9]. The uterine horn of *Bos taurus taurus* pubertal heifers should have a minimum diameter of 30 mm and a good tonicity [9,10], while *Bos taurus indicus* pubertal heifers should measure at least 25 mm in diameter [11].

Endocrinology - Folliculogenesis, Ovulation, Luteogenesis and Luteolysis

During the follicular phase of the estrous cycle, as the follicle increases its diameter, it becomes recognized as a dominant follicle [12] which secretes estrogen and inhibin [13]. The inhibin promotes a negative feedback under the adenohypophysis, decreasing the concentration of Follicle stimulant hormone (FSH) [14-16]. Making it insufficient for the development of the other subordinate follicles [17]. The dominant follicle becomes highly responsive to Luteinizing Hormone (LH) [16] and continues to grow. In contrast to the estrous cycle during which the follicles develop, the change in FSH dependence [18] for LH [19] occurs to the presence of LH receptors in the granulosa cells [20] and teak cells [21], allowing the growth of the dominant follicle in the environment with lower concentration of FSH [22].

The increasing of estrogen secretion by the preovulatory dominant follicle associated with decreased progesterone serum concentration, occurs to regression of the corpus luteum, induces hypothalamic secretion of GnRH [23]. GnRH induces the pre-ovulatory peak of LH [14]. After ovulation, LH at a concentration lower than that responsible for ovulation induces luteinization of follicular cells (granulosa and teak follicle cells), forming the corpus luteum [24]. Thus, FSH is the main responsible for the recruitment and selection of the dominant follicle, and the exposure of a pre-ovulatory dominant follicle to the frequencies of the LH pulses is the key to the final maturation and ovulation [25].

From ovulation, the luteal phase is characterized by an increase in the serum levels of progesterone secreted by the corpus luteum [26]. At this stage, even with a high progesterone concentration, follicular waves continue to occur. However, the dominant follicles that grow from each follicular wave regress, because progesterone determines negative feedback under the hypothalamus, blocking the peak of LH required for ovulation to occur [27].

The function of the corpus luteum is to produce progesterone during the luteal phase of the oestrous cycle for the maintenance of gestation. In addition, during pregnancy, progesterone prevents the secretion of gonadotrophins, preventing the occurrence of oestrus [28]. If on day 16 of the estrous cycle there is no maternal recognition of a gestation by interferon-tau secretion, regression of the

corpus luteum occurs by the action of prostaglandin secreted by the cow's uterus [29], decreasing serum levels of progesterone [30]. The prostaglandin secreted by the uterus, arrives at its place of action by the contra-current mechanism between the uterine vein and the ovarian artery [31].

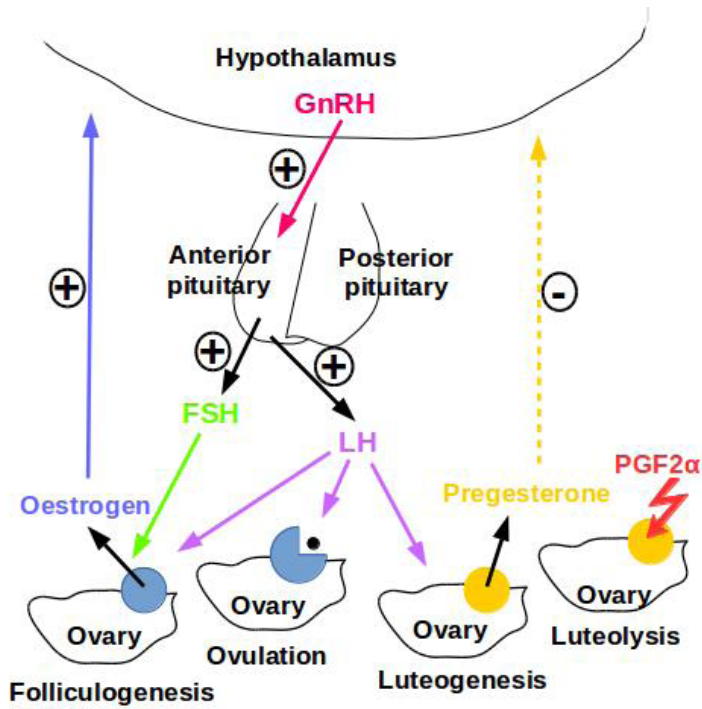

Figure 1: Schematic representation of the hypothalamic-pituitary-gonadal axis in the cow.

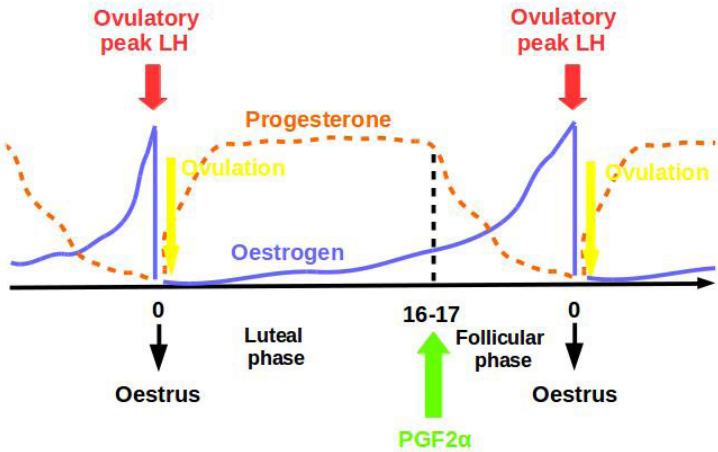

Figure 2: Schematic representation of the pattern of secretion of oestrogen, Luteinizing Hormone (LH), and progesterone in the oestrous cycle in cattle. and the pattern of growth of ovarian follicles during the oestrous cycle in cattle.

Oestrous Cycle of Cattle

Oestrous cycle is defined as the interval between two consecutive oestrus [32], being continuous and occurring at regular intervals. The mean duration is 21 days (18-24 days) in the adult female bovine, and the heifers present smaller intervals [33]. According to the dominant ovarian structure, this interestrus interval consists of two phases: the luteal phase (14-18 days) and the follicular phase (4-6 days). The luteal phase is the period following ovulation when the corpus luteum acts producing the progesterone and the follicular phase is the period from luteolysis to a

new ovulation, in which the dominant follicle produces the estrogen [34].

The estrous cycle is characterized by repeated ovarian changes with patterns of cell proliferation, differentiation and transformation including folliculogenesis, ovulation, luteogenesis, and luteolysis [35]. It occurs regularly throughout the year in the bovine species (annual polyester) and is only discontinued during gestation, lactation, severe malnutrition and pathological conditions associated with the persistent corpus luteum. The mechanisms by which day length may affect the reproductive function of cattle are not yet established, however most studies suggest that *Bos taurus indicus* is more influenced by photoperiod than *Bos taurus taurus* [36]. Similarly, nutrition can also significantly affect the reproduction of cattle. According to Rhodes et al. (1995, 1996), periods of reduced energy intake result in a lower circulating concentration of LH affecting growth and ovulation of the dominant follicle [37,38].

The regulation of the estrous cycle occurs through a series of hormones produced by the hypothalamus (Gonadotropin Releasing Hormone - GnRH), by the adenohypophysis (the gonadotrophins, Follicle Stimulating Hormone - FSH and Luteinizing Hormone - LH), ovaries (Oestrogen, Progesterone and Inhibin) and the uterus (Prostaglandin - PGF2α). These hormones interact with each other through mechanisms called positive feedback and negative feedback [39].

Stages of the Oestrous Cycle

The estrous cycle is divided into 4 phases: proestrus, oestrus, metestrus, and diestrus. In proestrus, under the synergistic action of pituitary gonadotrophins (FSH and LH), the follicle grows and gradually increases the secretion of estrogen until it becomes preovulatory follicle. At the beginning of oestrus, this preovulatory follicle secretes a large amount of estrogen responsible for changes in the genital tract and female behavior in oestrus [40]. Abundant vaginal mucus, vulvar edema, frequent urination and restlessness are evident signs that identify the female in oestrus [41].

The oestrus, period of sexual receptivity, lasts between 16 and 18 hours in *Bos taurus taurus* [42] and at around 10 hours in *Bos taurus indicus* [43], and presents the variation in expression between the cows and the breeds. Cattle beef females show a lower intensity of the signs of oestrus, which occurs more frequently during the night [43-46]. Environmental factors such as temperature and season of the year can influence the duration and intensity of the signs of oestrus manifested by taurine and cattle beef females [47,48]. During the warm months of the year, a decrease in the duration of oestrus occurs and increases the incidence of silent ovulation in taurine females [49].

The next step is the metestrus from the end of oestrus to the 5th day of the oestrous cycle [31,50] and it is when ovulation occurs, 24 to 48 hours after the starting of

oestrus or 10 to 16 hours after the end of oestrus [51,52]. In this way, the metestrus is considered a progesterone phase. Especially in heifers, occurs a pronounced capillary dilation of the endometrium because of the action of estrogen, rupture of uterine capillaries may occur, leading to the exit of bloody mucus 1 to 3 days after oestrus characterizing the metestrus hemorrhage [53].

The diestrus phase is a progesterone phase of the estrous cycle and is when the corpus luteum is functionally active producing progesterone, being responsive to the action of PGF2α. This phase runs from the 5th to the 17th day of the estrous cycle, being therefore, the longest duration phase. The corpus luteum is a temporary endocrine organ that is active during the phase of diestrus in cycling animals and during gestation. By the action of progesterone, the cervix becomes closed, the uterus becomes more flaccid, with less immunological resistance and greater activity of the glands [54].

Around the 15th day of the estrous cycle, when there is more activity of the corpus luteum, maximum values are observed in serum progesterone concentration [55-57]. According to Adeyemo and Heath (1980), during the luteal phase of the estrous cycle, higher concentrations of progesterone are observed in *Bos taurus taurus* females when compared to *Bos taurus indicus* [58]. This may be caused by the difference in the size of the corpus luteum [59-61]. Cattle beef female's corpus luteum meas-

ures around 15 to 21 mm while taurine's measures 24 to 27 mm [62-64]. According to Randel (1994), the smaller size of the corpus luteum of *Bos taurus indicus* is a result of a lower response to estrogen and the lower preovulatory peak of LH [65].

Follicular Waves

The heifer is born with 100,000 to 150,000 primordial follicles. Initiated the growth of the primordial follicle, it ovulates or suffers atresia [66]. Folliculogenesis is defined as the formation of preovulatory follicles from a pool of primordial follicles [67], and occurs through follicular waves. Each follicular wave is composed of three stages: recruitment, in which it involves the growth of a group of 5 to 20 follicles (cohort) ≥ 5 mm in diameter and is related to the increase in FSH concentration [14,18]; Selection, in which a follicle is selected; and of dominance when the selected follicle becomes dominant, reaches a larger diameter (15 mm) and inhibits the growth of the other subordinate follicles [68,69]. This dominant follicle ovulates if its degree of development coincides with regression of the corpus luteum or suffers atresia if it coincides with the presence of an active corpus luteum [39].

The number of follicular waves during the estrous cycle varies from one to four, being more common the cattle presenting two or three waves [70,71]. These changes occur in diet, management, milk production, lactation period, immediate postpartum [72] and duration of the

luteal phase of the estrous cycle [59,73,74]. In cycles of two waves, the first dominant follicle is anovulatory and the second, ovulatory. In the case of three waves, the first two dominant follicles are anovulatory, and the third develops until ovulation [75,76]. During the estrous cycle of *Bos taurus taurus*, the occurrence of two follicular waves is more frequent [76,77] and the majority of Nelore cows (*Bos taurus indicus*) present two follicular waves and The heifers three waves of follicular development per estral cycle [61].

For each follicular wave, a transient peak of FSH is required [78]. At the beginning of each follicular wave, approximately 24 small follicles (3-5 mm) are detected in *Bos taurus taurus* females [72]. However, in *Bos taurus indicus* females, this number of small follicles may be even larger. Buratini et al. (2000) detected up to 50 small follicles in the ovaries of Nellore heifers [79]. This higher population of antral follicles in cattle beef cows reflects the better results of embryo production in vitro [80] caused by the greater recovery of oocytes from these animals when compared to *Bos taurus taurus* cows [81]. Characteristics of follicular dynamics are similar between *Bos taurus indicus* and *Bos taurus taurus*, however, the dominant follicle of cattle beef females is smaller (10-12 mm) than that of taurine females (16-20 mm) caused by a lower follicular growth rate [61,76,82].

Follicular waves are always present in the ovaries of bovines with different reproductive conditions (prepuber-

tal period, gestation, postpartum, anestrus and during the diestrus phase). However, with the advancement of gestation, during the last 20 to 25 days of pregnancy, under the negative feedback exerted by progesterone, there is suppression of FSH release by the adenohypophysis, preventing the development of follicular waves [72]. As cattle are usually monovulatory, the presence of a single dominant follicle in the ovaries is more likely. However, the dominance of more than one follicle can occur in cows with high milk production [62,83,84], determining the occurrence of twin pregnancies.

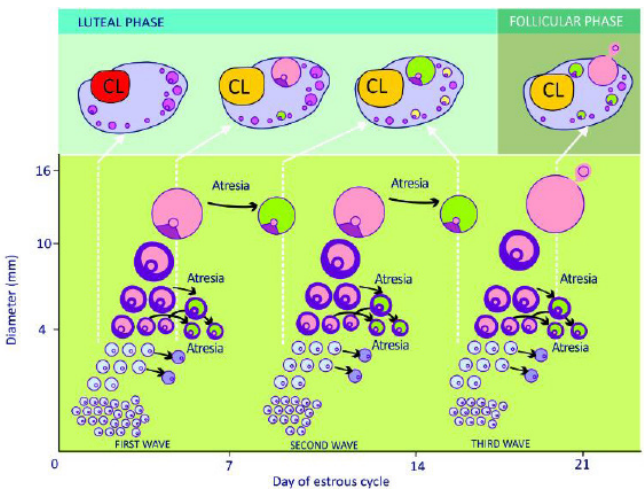

Figure 3: Schematic representation of follicular growth in cattle. Each wave of follicular growth is preceded by a transient rise in FSH concentrations. A surge in LH concentration occurs at the onset of oestrus and induces ovulation.

The ovaries act as the single unit, that is, each follicular wave includes follicles of both ovaries that respond in

synchrony and the presence of a dominant follicle in one ovary exerts an inhibitory effect on the other subordinate follicles in both ovaries [71]. However, a greater activity of the right ovary in relation to the left ovary is common [56,57,85].

Reproductive Physiology of the Bull

Puberty and Sexual Maturity in the Bull

Male puberty occurs when there is a production of the first ejaculate with at least 50 million spermatozoa with 10% minimal progressive motility [86], that is, when rapid testicular growth occurs, changes in the secretion of hormones and, as consequence, the onset of spermatogenesis [87].

Breeds differ in age and weight relative to puberty. Nellore bulls reach puberty by age 15 months [88,89], while bulls of European breeds reach puberty at 10 months of age [90]. Therefore, *Bos taurus indicus* animals present slower testicular development and tend to enter puberty later than *Bos taurus taurus bulls*. Feeding and thermal stress can contribute to the Schematic representation of the hypothalamic-pituitary-gonadal axis in these characteristics [91].

After puberty, the testicles of the bulls continue to grow and the number of spermatozoa in the ejaculate increases until 24 months of age, when they reach sexual maturity. At this time, there is an increase in seminal vol-

ume, progressive sperm motility, vigor and total sperm concentration, and a decrease in some sperm diseases [92-94].

Descent of the Testicles

Testicular development includes its descent from the abdominal cavity to the scrotal pocket through the inguinal ring. The descent of the testicles to the scrotum is preceded by the formation of the vaginal process from the peritoneum that internally coats the scrotum. At 140 days of gestation, the testicles of the bovine fetus are already housed inside the scrotum and completing the cellular differentiation [95,96].

Endocrinology and Spermatogenesis

The hypothalamus plays a key role in triggering puberty by stimulating the pituitary gland, which secretes increasing amounts of LH and FSH. Prepubertal males exhibit an amplitude of LH peaks around three months of age, resulting in differentiation, growth and proliferation of Leydig cells [90]. From puberty, testosterone levels begin to rise in the bloodstream and the bull begins to develop sexual organs, race characteristics and libido [97].

The two main functions of the testicles (testosterone secretion and sperm production) are controlled by pituitary gonadotrophins (FSH and LH), which in turn are controlled by the pulsatile secretion of the gonadotrophin releasing hormone (GnRH) from the hypothalamus [98]. The secretion of testosterone by Leydig cells, which is the

hormone responsible for libido in the bull, is controlled by the action of LH (luteinizing hormone). The decrease in LH concentrations coincides with the increase in testosterone secretion, which exerts a negative feedback effect on the hypothalamus, suppressing the production of GnRH and, therefore, LH. Each pulse of LH is followed about 1 hour later by a pulse of testosterone secretion [99] and the magnitude of the testosterone response rises with age [100].

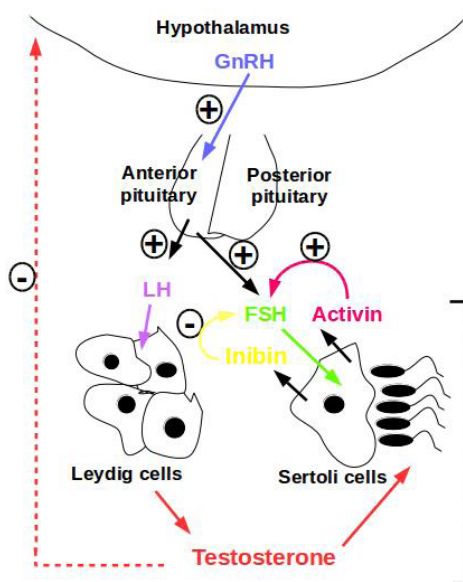

Figure 4: Schematic representation of the hypothalamic-pituitary-gonadal axis in the bull.

Spermatogenesis is controlled by FSH, LH and testosterone [101]. Sertoli cells are important for the control

of germ cell development, and synthesize the inhibin that suppresses FSH secretion and activin that stimulates the release of FSH by the adenohypophysis [102,103]. This process of sperm production is composed of two processes: spermatocytogenesis and spermiogenesis, and occurs within the seminiferous tubules [102].

Spermatocytogenesis is the development of spermatogenic tissue in the testicles, which occurs as a result of mitotic divisions of the spermatogonia present in the basal membrane of the seminiferous tubules [104]. As the cells divide, they move toward the lumen of the testicle lobe. Spermatogonia undergoes mitotic divisions forming primary spermatocytes that undergo meiotic divisions to form secondary spermatocytes. In turn, each secondary spermatocyte divides forming two spermatids.

In the phase of spermiogenesis, the spermatids undergo differentiation and each one will form a spermatozoon. Changes include the formation of the acrosome, the head, the intermediate piece and the tail [104]. The gradual release of the spermatozoa into the lumen of the seminiferous tubules is called spermiation. In the bull, the whole process of spermatogenesis takes about 62 days. Thus, a lesion in the testicles can interfere with spermatogenesis and sperm production, taking at least 2 months for sperm quality to return to normal.

After formation, the spermatozoa are transported by peristaltic muscle contractions through the epididymis, where they undergo additional maturation. This matura-

tion consists of the capacity of progressive sperm motility, changes in nucleus chromatin, loss of cytoplasmic droplet of the neck region, and maturation of the acrosome [105,106]. This occurs mainly in the head and body of the epididymis.

Spermatozoon mature and fertile are stored in the tail of the epididymis (105 20 GARNER, 1982), but after ejaculation in the female's reproductive tract, they must undergo a process known as sperm capacitation [107]. Which begins in the cervical mucus and continues during the uterine transit and in the oviduct [108]. Sperm capacitation is related to the change in the motile pattern leading to hyperactivation, as well as changes in the plasma membrane allowing the occurrence of the acrosomal reaction phenomenon [109].

When ejaculation does not occur, most spermatozoa are eliminated in the urine [110] or may undergo gradual senescence, losing motility and fertilization capacity. The ejaculations obtained after a period of sexual rest usually contain a high percentage of dead spermatozoa.

Testicular Parameters and Semen Production

Sperm production is related to testicular size. Characteristics referring to scrotal circumference, volume, weight and testicular shape are parameters used in the selection of breeding [111,112]. Only scrotal circumference is not the representative measure of sperm production, and testicular volume can be a further measure to represent this production [111,113,114].

Testicular size and scrotal circumference are influenced by age and breed. Mature bulls with longer time in sexual activity have a larger scrotal circumference with a greater amount of testicular parenchyma, increasing the testicular weight [115]. In older bulls, caused by the possibility of fibrous tissue being present in the parenchyma of the testicles, scrotal circumference may not predict the animal's actual sperm production capacity [116].

According to Bailey et al. (1998), the longer testicles have a greater surface area of contact with the environment, which facilitates thermoregulation caused by the uniform distribution of blood vessels and sperm tissue [117]. Thus, the more elongated testicular forms are more advantageous to reproduction. Animals with the same scrotal perimeter, but with different testicular lengths, present a larger testicular volume the longer the length and therefore, the greater sperm production [94].

As other double organs of the body, both sides of the testicles and epididymis of bulls show developmental similarity, morphology, and physiological function during the growth of the animal [114-115,118].

There is a direct relation between the scrotal circumference and the physical and morphological characteristics of the semen [119]. According to Randel (1994) and Pastore et al. (2008), the scrotal circumference shows a positive correlation between motility and sperm vigor [33,118]. And as bulls reach sexual maturity, semen quality improves. The changes in spermatic morphology tend

to present a continuous decrease caused by the maturation of the epithelium of the seminiferous tubules and stabilization of the seminal characteristics [89].

Testicular Thermoregulation

For a testicular function to be satisfactory, the temperature of the testicles in the scrotum should be maintained at 2 to 6°C below body temperature. The maintenance of physiological testicular temperature depends on factors that involve the scrotum, pampiniform plexus, spermatic cord and internal vascularization [120].

The countercurrent mechanism refers to the loss of temperature of the testicular artery by lowering blood pressure as it enters the testicular parenchyma [121]. This occurs by testicular veins that surround the arterial branches helping the temperature drop. Failures in testicular thermoregulation favor testicular degeneration, leading to increased morphological defects of spermatozoa, reduced seminal quality and fertility [122].

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Chapter 03

Regulation of the Hypothalamic-Pituitary-Gonadal Axis and the Manipulation of the Estrous Cycle of Bovine Females

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Abstract

The hormones are used in the induction and synchronization of estrus and ovulation of females for Artificial Insemination and other biotechnologies. Gonadotropin releasing hormone is an important regulator of the release of follicle stimulating hormone and luteinizing hormone, acting on the reproductive regulation according to the phase of the estrous cycle. Pituitary hormones are directly involved in the ovarian and follicular dynamics of the estrous cycle. Hormones such as estradiol and progesterone are released by ovarian structures. Estradiol is secreted by internal teak cells, while progesterone is released by the luteal cells of the corpus luteum. The uterine endometrium is involved in the regulation of the estrous cycle of the bovine female. There are protocols that use progesterone as a basis to simulate the effects of the corpus luteum, preventing the release of LH pulses. The use of them demonstrate most efficiently used in all reproduction techniques, including the induction of puberty in heifers.

Introduction

Breeding superior genetic animals is a major challenge for beef and dairy farming. The diffusion of genetic material is accelerated with the application of reproduction biotechniques. Examples of biotechniques are artificial insemination (AI) or fixed time artificial insemination (FTAI), superovulation (SOV), ovum pick-up (OPU), *in vitro* embryo production (IVEP) and transfer of embryos (ET) or fixed time embryos transfer (FTET).

The manipulation of the estrous cycle of the females allows the veterinary professional to optimize the genetic material by increasing the number of products throughout the reproductive life of genetically superior females [1]. The natural hormones or synthetic analogous used to manipulate the estrous cycle are intended to restart a new cycle, assist in follicular growth and induce ovulation. Consequently, the application of protocols using these hormones provides the induction and synchronization of estrus, being applied individually or in groups of cyclic or acyclic females.

The hormone therapy has facilitated the application of reproduction biotechnologies, being used in the induction and synchronization of estrus and ovulation of females for AI and FTAI, and of recipients in ET and FTET; with induction of follicle growth simultaneously and induction of ovulation in superovulation donors; and the resumption of the cycle to obtain follicular waves in emergency for the OPU, recovering a greater number of oocytes for the IVP. Hormones are classified as analogous of estradiol, prostaglandins, gonadotrophins and progesterone and have different functions.

The hormonal protocols consists of different combinations of the effects of natural or synthetic hormones promoting the manipulation of the estrous cycle of the bovine female with the objective of using reproductive biotechnologies. The objective of the protocols is to simulate the events occurring in the distinct phases of the estrous cycle, optimizing the reproductive process.

Hypothalamic Hormones

Gonadotropin releasing hormone (GnRH) is an important regulator of the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), acting on the reproductive regulation according to the phase of the estrous cycle. The physiological release of GnRH occurs in response to circulating levels of estradiol and progesterone. GnRH acts by stimulating the pituitary in the release of FSH and LH [1].

The regulation of GnRH release occurs through feedback mechanisms involving the hormone levels of gonadotrophins (FSH and LH) and steroids (estradiol and progesterone). Feedback systems can be considered positive (stimulatory) or negative (inhibitory), many feedback systems are observed in the hormonal regulation of the hypothalamus-hypophysis-gonadal axis and are addressed in the next topics [2,3].

Gonadotrophin Releasing Hormone

Synthetic GnRH (buserelin acetate or gonadorelin acetate) is applied in protocols with two objectives: induction of follicular growth and induction of ovulation [4]. Its action occurs at the pituitary level, stimulating the release of FSH or LH. Induction of follicular growth occurs in the presence of small follicles present in the ovaries.

Physiologically in the presence of progesterone secreted by the corpus luteum (CL) GnRH induces the pituitary to release FSH, stimulating follicular growth. However, when the follicle is in the preovulatory stage, basal levels of progesterone and high levels of estradiol are found. At that time, we find a typical physiological example of positive feedback from the axis, where the high level of estradiol stimulates the hypothalamus to the release of GnRH for the occurrence of pituitary LH peak inducing ovulation.

Synthetic GnRH is used in hormonal protocols at different times, according to the objective proposed in the protocol, being applied alone or in association with progesterone devices.

Pituitary Hormones

Pituitary hormones FSH and LH are directly involved in the ovarian and follicular dynamics of the estrous cycle. FSH stimulates follicular growth in the presence of luteal levels of progesterone.

The peak of LH induces ovulation, its release is directly associated with steroid levels such as estradiol and progesterone. The follicles are ovarian structures responsible for the production of estradiol. As the follicle grows by FSH stimulation higher levels of circulating estradiol appears [2].

At this moment two mechanisms of negative feedback are observed. The first corresponds to the inhibition of GnRH release in the hypothalamic center due to the increase in FSH levels. The second inhibition of FSH release is a result of the increase in circulating estradiol level.

A positive feedback mechanism is also observed, an increase in estradiol levels that stimulates the occurrence of LH peak in estrus to induce ovulation in metaestrus in cattle, with estrus being a stage of estrogenic predominance and basal levels of progesterone.

Follicle Stimulating Hormone

Analogous FSH hormones are natural, extracted from the swine pituitary gland (FSHp) or the blood of pregnant mares (equine chorionic gonadotrophin - eCG). FSH analogous act on the hormonal protocols promoting follicular growth, acting directly on follicular FSH receptors. For its action to be effective the application must be associated with the presence of a progestogen or corpus luteum, where progesterone is in luteal levels.

In the protocols these hormones are used with the objective of promoting follicular diameter and follicular maturation in techniques such as AI and FTAI, and as a promoter of the simultaneous growth of several follicles in techniques such as SOV.

Luteinizing Hormone

Luteinizing hormone extracted from the swine pituitary (LHp) and human chorionic Gonatrophin (hCG) are examples of LH analogous. They act by binding to the LH receptors on the follicular wall, their functions correspond to the final maturation and the induction of follicular ovulation. Like the endogenous LH peak, their analogous induces ovulation of preovulatory follicles.

In bovine females the peak of LH is observed shortly after the estradiol peak during estrus and ovulation occurs in the metaestrus, different from the other species where both events occur during estrus [1,4].

Hormones Secreted in the Gonads

Hormones such as estradiol and progesterone are released by ovarian structures. Estradiol is secreted by internal teak cells, while

progesterone is released by the luteal cells of the corpus luteum. Both are important regulators of the release of gonadotrophins (FSH and LH).

In the presence of endogenous progesterone, synthesized in the corpus luteum, the hypothalamic-pituitary axis is blocked. At luteal concentrations progesterone inhibits estrus manifestation, peak LH, and ovulation.

Consequently, in the presence of progesterone, FSH acts to stimulate follicular growth. As the follicle reaches diameters greater than 6 mm an evident increase in estradiol secretion is observed. With increasing follicular diameter and circulating estradiol, it is observed that smaller follicles that were recruited in the same wave pass through the process of follicular atresia. Consequently, elevation of estradiol levels promotes positive feedback, stimulating luteolysis of the corpus luteum and release of the LH peak.

Ovulation of the preovulatory follicle occurs after the peak of LH. With the occurrence of ovulation, the space previously filled by the ovarian follicle is replaced by the corpus luteum. This is responsible for the production of progesterone [2].

Progesterone is one of the important regulators of the hypothalamic-pituitary-gonadal axis and responsible for maternal recognition and maintenance of pregnancy for preparing the reproductive tract for embryo implantation.

Estradiol Analogous

Examples of estradiol analogous are: estradiol benzoate (EB), estradiol valerate (EV), 17 estradiol and estradiol cypionate (EC). The role of estradiol analogous depends on the time at which it is applied in the hormonal protocol, acting as in follicular atresia, associated with high plasma concentrations of progesterone, or as inducer of estrus and ovulation, associated with low plasma concentrations of progesterone [5,6]. According to the moment of application, it will

perform one or another function. To demonstrate the effect of follicular atresia of the developing wave on the ovary of the female, the estradiol analogous should be associated with the progestogen.

When used as a stimulator of LH peak release, it should be applied at the time or after removal of the progesterone analogous and luteolysis of the corpus luteum present in the female ovary [5,6]. The time of action of EB (24-36 hours) and EC (48-72 hours) is associated with the number of treatment adopted in the protocols because of the interval between the application of the analogous and the release of the LH peak [6].

Progesterone Devices

Progesterone is pharmacologically related as slow release mechanisms in the form of atrial or intravaginal devices impregnated with natural progesterone or medroxyprogesterone acetate. Currently the protocols adopt periods between 7 and 9 days of progesterone device [1,5]. Progesterone is commonly used in the form of intravaginal devices, increasing in a few hours the concentration of circulating progesterone at supraluteal levels, blocking the hypothalamo-pituitary-gonadal axis, impeding the pulsatility of LH [5].

With the removal of the progesterone device the manifestation of estrus occurs from 48 to 72 hours, the application of the associated estradiol analogous or 24 hours after device removal is related to the luteolytic action of estradiol, which potentiates the effects of oxytocin and prostaglandin increasing the frequency and amplitude of uterine contraction.

Hormones Secreted in the Uterus

The uterine endometrium is involved in the regulation of the estrous cycle of the bovine female. In the uterine endometrium are found the receptors of oxytocin, which is a hormone produced by the hypothalamus and stored in the posterior lobe of the pituitary gland.

Oxytocin is a stimulator of uterine contraction, facilitating the transport of spermatozoa acting in synergism with estradiol.

Physiologically, between 14 and 16 days after ovulation, the uterus that did not recognize pregnancy, due to non-fertilization and embryo formation, prepares to start a new cycle, closing the luteal activity through the release of prostaglandins such as PGF, that has luteolytic action at the ovarian level, inducing luteolysis. The release of PGF is stimulated by estradiol and its action on myometrial contraction [7,8].

In the occurrence of fertilization the embryo formed releases interferon-τ inhibiting the activity of PGF, being this one of the main mechanisms of recognition of pregnancy [7,8].

Prostaglandin Analogous

The main activity of prostaglandins is to promote luteolysis, with regression of the luteal activity of the corpus luteum and consequent reduction of plasma concentration of progesterone [1,9]. Luteal inactivity by regression of the corpus luteum culminates in the release of the hypothalamic-pituitary-gonadal axis, which in the presence of estradiol stimulates the final growth of the dominant follicle [10].

With the released axis the female enters the proestrus and estrus, manifesting the characteristic sexual behavior [1]. The corpus luteum responds to prostaglandin administration only after luteinization, which occurs after four days of ovulation and is not responsive during the period of CL formation in the metaestrus [10].

Cloprostenol sodium, D-Cloprostenol and Dinoprost tromethamine are analogous molecules of PGF.

Hormonal Protocols

When combined hormones and their analogous have complementary functions, presenting action in females in the different phases of the estrous cycle. The hormonal combination is called the hor-

monal protocol. In choosing the protocol, characteristic points such as subspecies (*Bos taurus* or *Bos indicus*), productive ability (beef or milk) and female body score should be observed.

The protocols are elaborated according to the objectives, the hormones and their analogous being applied according to the desired activities in each moment. The use of hormones and their analogous has limitations, since they are regulated by the national health agencies in the different countries. Depending on the country, some restrictions are applied, preventing the use of certain protocols.

In general, the protocols can be divided into three distinct moments: follicular wave restart, follicular growth and dominance, and ovulation induction.

Protocols with Progesterone

They are protocols that use progesterone as a basis to simulate the effects of the corpus luteum, preventing the release of LH pulses. The protocols for the use of progestogens used in FTAI are divided into protocols of three (Figure 1) and four treatments (Figures 2, 3 and 4). These protocols present rates of 70 to 90% of estrus manifestation.

Intravaginal devices are pharmacological displays of devices impregnated with 0.5 to 1.9 g progesterone. The devices may be 1 or 3 uses according to the concentration of impregnated progesterone. The release of progesterone should be slow, maintaining luteal or supraluteal levels [11,12].

The dose of the implant is the main care in the protocols using progesterone. Nulliparous or primiparous females can not receive high concentrations of progesterone, as new implants of 3 uses, being recommended for these females monodose implants or in the 3rd use of the implant. For multiparous females, any implant model can be used; in dairy cows the use of implants with high concentration

of impregnated progesterone is of choice in view of the high hepatic metabolism in these animals.

The protocols presented in the figures were adapted from the literature [1-5,10-13].

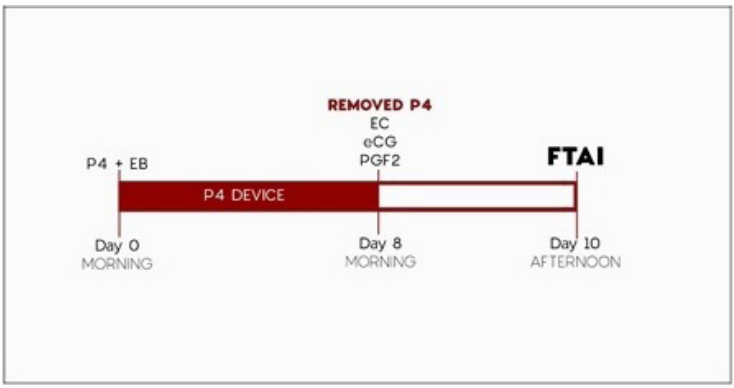

Figure 1: Protocol with progesterone for FTAI in three treatments.

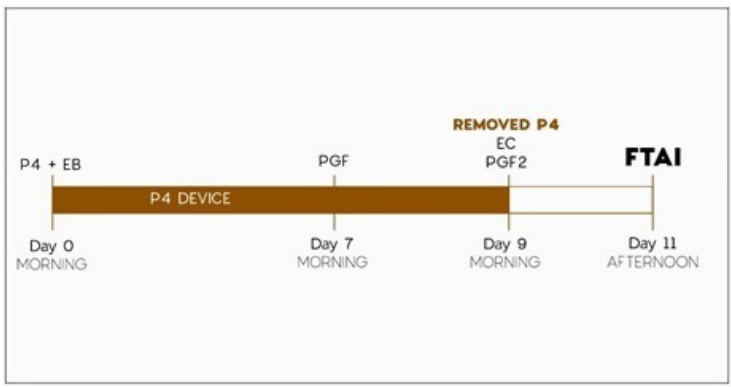

Figure 2: Protocol with progesterone for FTAI with pre-luteolysis, in four treatments.

The rates of application between 2 and 5 mg estradiol analogous associated with progesterone implant placement on D0 is to promote existing follicular atresia wave, regardless of the stage of follicular development [9].

When plasma estradiol and progesterone levels are high, they simultaneously inhibit the release of gonadotrophins FSH and LH causing follicular atresia.

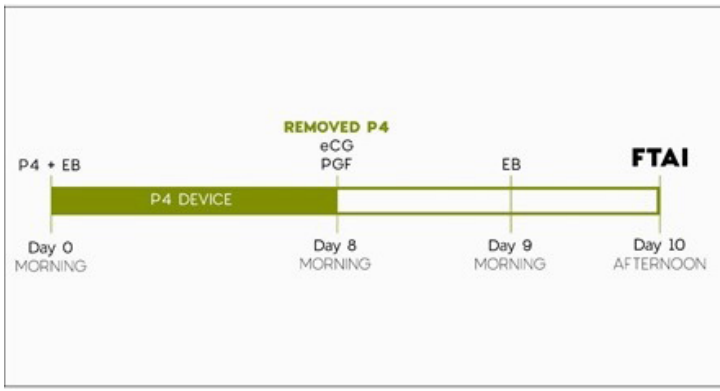

Figure 3: Protocol with progesterone for FTAI in four treatments.

With the reduction of plasma levels of estradiol and maintenance of progesterone levels, the synchronization and emergence of a new follicular wave occurs 3 to 6 days after the initiation of the protocol [14].

In the synchronized follicular wave the follicle will reach dominance and ovulation will occur at a predetermined time. Any of the estradiol analogous at D0 can be used, differing only in the time of onset of emergence of the new wave.

Among the analogous, EB and EC are those commonly used. Very high levels of progesterone are not desired, especially in heifers,

since they inhibit the release of gonadotrophins, preventing a correct development of the follicular wave [14].

Between D3 and D8 of the protocol will occur the emergence of the new wave, with recruitment, divergence, selection and follicle dominance. For the occurrence of ovulation, the removal of the implant and application of prostaglandin analogous in D8 are performed to reduce the levels of circulating progesterone, unlocking the hypothalamic-pituitary-gonadal axis. In D8 the association of fast-acting (EB) or slow-acting estradiol analogous (EC) will depend on the number of treatments adopted in the protocol, EB (action time 24-36 hours) being adopted in four-hand protocols and EC (action time 48 to 72 hours) for three treatments protocols due to the time of action of the analogous. The dose of estradiol analogous employed in D8 is similar to that of D0 [13,15].

Estradiol analogous has the function, at this point in the protocol, to induce the release of LH due to the low concentrations of progesterone. Estradiol is also important in the expression of oxytocin receptors in the endometrium, inducing contraction of the myometrium to release prostaglandin for regression of the corpus luteum [7].

The prostaglandin analogous is applied in the protocols associated with removal of the progesterone implant in D8.

Luteolysis occurs after the application of PGF by unblocking the axis, since removal of the implant has already occurred [9]. The release of the axis allows the increase of plasma levels of estradiol produced by the dominant follicle and consequently the female enters the proestrus and estrus, being able to perform the AI. In protocols using progesterone the estrus manifestation occurs between 24 and 96 hours after the application of prostaglandin. The dose used ranges from 0.1 to 13.0 mg of prostaglandin analogous.

In some hormonal protocols a pre-luteolysis is observed, performed in an attempt to simulate endogenous prostaglandin release and reduce progesterone levels [1].

Equine chorionic gonadotrophin (eCG) is commonly used in hormonal protocols, aiming to increase the follicular diameter and consequently the corpus luteum formed after ovulation, increasing the rate of conception. The eCG promotes the final maturation of the follicle by binding to the FSH and LH receptors. The recommended dose in FTAI protocols is 300 IU [13].

By convention, the hormones used in the protocols should be applied in the morning, in view of the managements that occur in the farm. Fixed-time insemination occurs at the beginning of the afternoon, respecting the application of the estradiol analogous adopted in the protocol (EB or EC).

Protocol with Gonadotropin Releasing Hormone (GnRH)

Protocols that use GnRH are a cheaper protocol alternative, not using progesterone. Two applications are required, being D0 and D9.

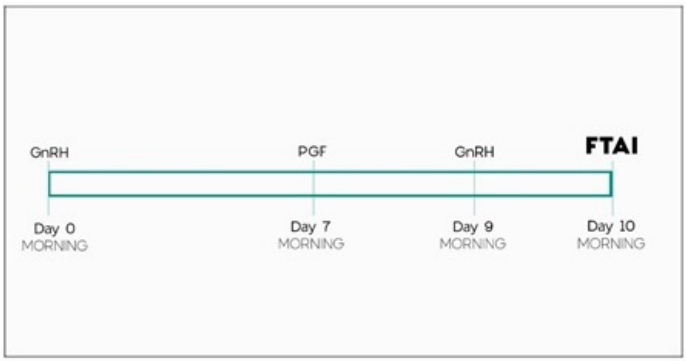

Figure 4: Protocol with analogous of GnRH for FTAI in four treatments.

In D0 the function of GnRH is to stimulate follicular growth or ovulation depending on the diameter of the follicle in the ovaries. In the occurrence of ovulation induced by the application of GnRH in D0, the formation of a new corpus luteum will occur, blocking the hypothalamic-pituitary-gonadal axis. With the blocked axis there is an increase in the release of FSH, promoting the development of a

new follicular wave until the dominance stage. In the case of females, where the corpus luteum is already functional in the ovary, the GnRH dose will stimulate the follicular development of the growing wave [4-5,16].

In D7, the application of prostaglandin (0.1 to 13.0 mg) will induce luteolysis, releasing the axis, allowing the increase of levels of estradiol secreted by the dominant follicle. The high concentrations of circulating estradiol induce the female to estrus.

The second application of GnRH occurs in D9, inducing ovulation of the preovulatory follicle. Insemination should be performed on D10 (early afternoon), or 36 hours after the application of the second dose of GnRH [4]. The use of this protocol does not require the observation of estrus, but for small herds can be applied with estrus observation. The dose of GnRH ranges from 25 to 100 μg in D0 and D9.

In conditions where the objective is not the synchronization of the females, the application of a dose of GnRH helps to promote ovulation, however it is necessary for the female to be observed in the estrus for the application to be performed.

Protocol with Prostaglandin

Protocols with prostaglandin analogous are applied to cyclic females with corpus luteum. The isolated use of prostaglandin analogous is performed in protocols requiring the observation of estrus occurring between 48 and 72 hours after administration of the prostaglandin dose [17-19].

Differently from the progesterone protocol, when prostaglandin analogous are applied alone in a single dose, the estrus manifestation in the herd ranges from 20 to 50%. Observation of estrus should occur at 12-hour intervals, with the female inseminated after 12 hours from the onset of estrus manifestations.

Increased use of estrus occurs when two doses of prostaglandin are applied in D0 and D11. The use of two applications promotes estrus synchronization in a group of bovine females [20].

Induction of Puberty and Hormonal Protocols in Heifers

Heifer puberty is achieved by presenting fertile cycles with gestation capacity. For cycles to be fertile, with ovulation occurring, prepubertal heifers need to trigger a series of events that culminate in an increase in ovarian diameter and the release of pulses and peak of LH. Once ovulation occurs the female reaches puberty and follicular wave growth will occur at the rhythm of the estrous cycle. Once ovulation occurs the female reaches puberty and follicular wave growth will occur at the rhythm of the estrous cycle [3].

Once ovulation occurs, the first corpus luteum is formed, which is a small length of the corpus luteum, but important for endometrial stimulation.

The induction of puberty is an important tool to reduce the age at first calving; however it is necessary to take into account the age, size and uterine development of young females in order to achieve good results.

The results of prepubertal females in FTAI protocols are unsatisfactory, however, induction allows the female to reach puberty and the next cycles being regular increase the chances of pregnancy. The pre-synchronization of prepubertal heifers to the low concentration progesterone implant for 8 days, followed by the application of 1.0 mg EB in D9, increased the number of females that reached puberty and gestation capacity when the FTAI was performed after pre-synchronization.

An intravaginal device with high concentrations of progesterone impairs follicular growth in heifers. It is indicated for the protocols of estrus and ovulation synchronization (FTAI and FTET) of heifers, one use devices or the 3rd and 4th use of 3-use devices.

In the use of one-use devices, the association of prostaglandin analogous at the dose of up to 0.1 mg should occur at the D0 adjacent to the intravaginal implant application.

The protocol for heifers should be associated with the use of prostaglandin analogous in the D0 with the application of the implant and EB, reducing the negative effects of high concentrations of circulating progesterone through partial luteolysis of the corpus luteum maintaining luteal levels of progesterone, not allowing LH pulsatility without interfering with follicular growth and ovulation capacity of the preovulatory follicle. The use of this protocol does not require observation of estrus.

Receptor Preparation Protocols for ET or FTET Programs

In protocols for embryo recipients, instead of performing insemination, the embryo is inoculated 6 to 7 days after ovulation (Figure 5). In ET and FTET the objective is for the recipient female to manifest estrus and to have ovulation synchronized for later embryo. The recipient's uterine age should be synchronized with embryonic age.

The protocols showed in Figures 1 and 3 are advocated and the TETF performed on D16 replaces the IATF of D10, as shown in Figure 5. It is important to emphasize that in the case of pubescent heifers are used as recipients, the protocol applied to heifers should be used.

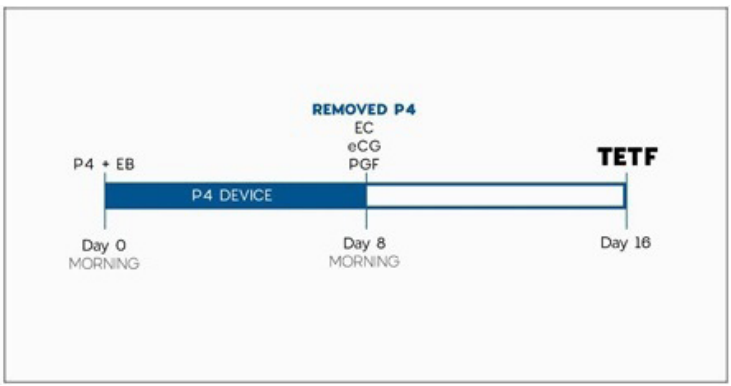

Figure 5: Receptor protocol for FTET.

The same protocols can be applied with observation of estrus in recipients. In this case the estrus observation should occur every 12 hours, over a period of 72 hours, starting 24 hours after the removal of the progesterone implant.

The preparation of the receptors can take place to transfer the embryos produced in vitro, in which case the transfer occurs in D17 due to the accelerated growth of the embryo.

Protocol for Superovulation of Embryo Donors

Donors are females of high genetic potential of zootechnical interest. The superovulation program allows obtaining several donor embryos in a single cycle. In the SOV protocol (Figure 6), the association of progestogen and EB is used for the synchronization of the new follicular wave as in the protocol for FTAI.

In the absence of progesterone the estrus can be used as the basis for the superovulation procedure. For this, it is enough to identify the estrus of the donor and consider the day of the estrus as D0.

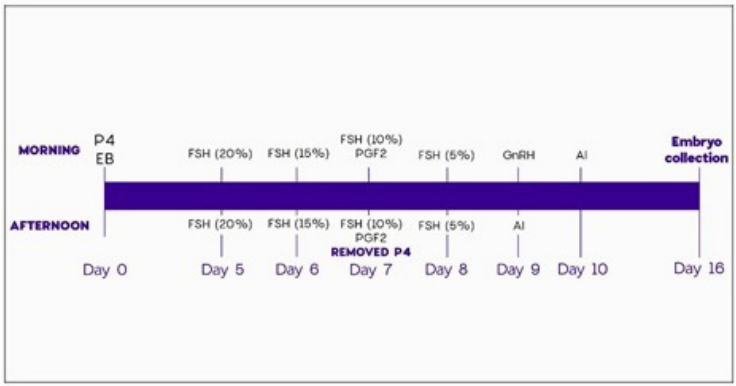

Figure 6: Protocol of superovulation of donors.

As in FTAI the emergence of the new follicular wave after the reduction of BE levels starts approximately on the 4th day. At which time the application of gonadotrophins (FSHp) is initiated [21].

At 12 hours intervals the FSHp is applied on days 5, 6, 7 and 8 of the superovulation protocol. This frequency occurs due the half-life of this molecule. Doses are applied in a decreasing manner, simulating what happens physiologically [22].

In D7, two doses of prostaglandin analogs (morning and afternoon) are used to promote luteal corpus luteolysis, associated with withdrawal of the progesterone implant and release of the hypothalamic-pituitary-gonadal axis [18,21].

In D8, the last two doses of FSHp are applied, multiple follicles will be present in the ovaries in the diameter of dominant follicles, reaching the preovulatory stage after the removal of the progesterone implant.

In D9, the GnRH analogous is used to induce ovulation of multiple follicles. In the donor, two inseminations are performed, being D9 (afternoon) and D10 (morning).

Wash the uterus and collect the embryos occur on D16, the day on which the embryos are transferred to the recipients.

For progesterone analogous, both EB, prostaglandin and GnRH are recommended in the same doses and care as the FTAI protocols presented above.

The dose of FSHp depends on the unit of measurement of the drug, ranging from 120 to 200 mg or 250 to 500 IU [22].

The mechanism of drug administration in decreasing doses follows the same pattern independent of the unit of measurement, being D5 (40%), D6 (30%), D7 (20%) and D8 (10%) of the total dose, divided into two applications in the day [21].

Dose selection should take into account the animal category (nulliparous, primiparous, multiparous), body score, subspecies, breed and productive ability of the donor.

Animals with high body and milk scores present rapid metabolism of the FSHp molecule, requiring higher doses. On the other

hand, cows with low body score may present exacerbated responses to high concentrations of FSHp.

Nulliparous females require lower doses for gonadotrophin superovulation compared to multiparous females due to the high availability of available FSHp receptors.

The doses differ between dairy and beef animals. It is known that SOV programs are satisfactory in all races, considering an adequate management and number of protocol replications in the same donor.

It is recommended to perform three programs with 60-day intervals in each donor, followed by progesterone rest, which occurs with gestation in the donor's womb, in order to avoid a reduction in the superovulatory response of the donor.

Individual responses are observed between animals of the same breed and family, requiring prior selection of animals that present satisfactory responses.

Conclusion

The applications of hormonal protocols should consider the maximum use of estrus in a commercial herd of cattle.

The use of protocols with progesterone implants are the most efficiently used in all reproduction techniques, including the induction of puberty in heifers. Hormonal protocols are an efficient alternative to concentrate births of calves, reduce intervals between generations and allow the application of reproductive biotechniques, accelerating genetic improvement.

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Chapter 04

Hormonal Applications in Horse Reproduction

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Abstract

Horse reproduction is not only a science, but it is also an art. It leads us to a different reproductive approach from what is used in other farm animals. It takes much more than hormonal protocols to manipulate horse breeding, but a deep knowledge of the reproduction physiology, hormonal actions, commercial preparations and a touch of sensitivity that will allow an effective individual intervention and good results. Hormonal applications are essential to biotechnologies programs implantation in the horse. In this chapter, hormones and its applications into a variety of uses and possibilities for the horse reproduction practice will be discussed and presented.

Introduction

Horse reproduction is not only a science, but it is also an art. Mares and stallions are not selected by reproductive characteristics, which makes a huge difference from other farm animals. The motivation for reproducing a horse is so specific and most of the time it may be driven by passion. These lead to a different reproductive approach and take much more than hormonal protocols to manipulate horse breeding, but a deep knowledge of the reproduction physiology, hormonal actions, commercial preparations and a touch of sensitivity that will allow an effective individual intervention and good results. I use to compare horse breeding to human breeding because no matter what problem is detected, no matter what age the animal is, our work is toward the obtainment of one or more healthy products.

There are many hormonal applications in horse reproduction for mares and stallions. The main uses in mares include the anticipation of cyclicity, ovulation induction, luteolysis, ecbolic effect, superovulation and acyclic recipient mare preparation. Hormonal applications in stallions are used to promote libido increase, descent of the testes and ejaculation. The decision of why, what moment and what hormone will be used is based on the physiologic knowledge. The aim of this chapter is to discuss the hormones, its function, applications and results.

Hormones

Different hormones may be used for different purpose and moments. To decide the establishment of a treatment it is important to know some characteristics of each hormone and commercial preparations. The hormones used in the equine reproduction routine are estrogens, progestagens, prostaglandins, oxytocin, gonadotrophins, sulpiride and GnRH analogues.

Steroid Hormones

Steroid hormones are derived from cholesterol and formed by a common structural nucleus. Even though steroid hormones show different functions, they differ from each other basically by the number of carbon atoms: a steroid of eighteen carbon atoms shows estrogenic function, while a steroid of nineteen carbon atoms shows androgenic function and a 21 carbon atoms shows a progestagen activity. Cholesterol, a 27 carbon atoms steroid, may convert in pregnenolone (20 carbon atoms) when its lateral chain is cleaved and it is subsequently converted to progesterone, which may be converted to androgens and estrogens.

All cells that synthetize steroid hormones use the same biossynthetic pathways differing only by the enzymatic system. In females, estradiol is the steroid hormone predominantly produced by the follicular granulosa cells, while progesterone is produced by the luteinic cells during diestrous. Androgens are the predominant steroid hormones produced by the testes, however, it is important to emphasize that in stallions high concentrations of estrogens are found in the testes produced by the sertoli cells [1].

Most of the circulating steroid hormones are coupled to albumin, a plasmatic protein of low affinity and high capacity to steroids. The other part of steroid hormones is coupled to one or more specific proteins of high affinity. These binding proteins affect steroid half-life and rate of elimination.

Estrogens, progestagens and androgens are steroids that act altering gene expression. Steroid hormones are produced by the gonads, genitalia are their target tissues, they promote secondary sex characteristics and feed back to hypothalamus inhibiting GnRH pulses and therefore both LH and FSH [2].

Estrogens

Estradiol 17β (E2) is a primary biologically active estrogen produced by the ovaries. Estrone and estriol also represent other metabolically active estrogens. In the follicle, estrogens are produced by the granulosa cells where the androgens produced in teca cells are aromatized into estrogens. Most of ovarian estrogens are secreted during estrous promoting estrous behavior and morphofunctional changes.

Maturing follicles produce E2 that increases as follicles develop. In the hypothalamo-pituitary-gonadal axis, the E2 increase close to dominant follicle selection, and in association to inhibin produced by follicles, is responsible for lowering FSH secretion that promotes subordinate follicles atresia [3,4]. During dominant follicle development and maturation, the increase in E2 secretion shows a positive feedback on LH pulsatile secretion [5,6], however, high estrogens levels may lead to a suppression on LH secretion.

Important uterine modifications are necessary to warrantee female fertility and estrogens actions are responsible to part of its modifications. Estrogens induces increase vascularity and leucocytes infiltration, progesterone and estrogen receptors increase [7] and induces decreased uterine and cervical tonus. It is commonly used to induce estrous behavior and as part of acyclic recipient mares protocols [8,9].

There are three estradiol esters commercially available: benzoate (BE), valerate (EV) and cypionate (ECP), hey differ in pharmacokinetic properties. While E2 half-life is about one day, among the commercially available esters, BE shows the shorter half-life (three days), followed by EV (seven to nine days) and ECP (ten to twelve days).

However, recent studies in mares have shown that the administration of a single dose of 2.5 mg of EB to anestrous mares produces similar estrogen concentration to that found in cyclic mares [10]. A longer activity of EB in the uterus than it was so far expected has also been observed by non-published data from our laboratory from Unesp campus Botucatu, SP, Brazil, what shows how scarce our knowledge about estradiol pharmacokinetic in mares is. The lack of knowledge leads to inconsistent protocols and doses determinations, which are most of the time defined by the extrapolations of trials carried out on cattle. These explain why empirical estradiol doses and frequency of injections are routinely used and an enormous variety of protocols has been reported and will be discussed further in this chapter.

Also, stallions interesting show high concentrations of estrogens, especially estradiol, in the testis. Estradiol seems to be involved with the rise up in circulating LH and spermatogenesis, a role designated to testosterone in other animal species [11,12].

Progestagens

Progesterone (P4) is the progestagen of higher prevalence during estrous cycle. It is produced and secreted luteinic cells. Many other progestagen may be metabolized from P4, especially in pregnant mares when the placenta begins to produce progestagens on day 70 of gestation. Progestagens are essential to pregnancy maintenance and for altering endometrial function [13], for controlling endometrial differentiation and local immune response to render the uterus receptive and allow embryo implantation [14], for inhibiting myometrial contractility [15], for increasing uterine and cervical tonus and for altering endometrial and myometrial estradiol and progesterone receptors expression [9].

In the hypothalamo-pituitary-gonadal axis, the P4 secretion by the corpus luteum (CL) during diestrous inhibits the preovulatory LH surge, consequently, inhibits ovulation [2,16,17,18].

Progestagens show an important role in the preparation of the uterus to receive the embryo and to maintain gestation. For this rea-

son, progestagens are commonly used for preparing acyclic recipient mares [8,9] in embryo transfer (ET) programs. Some commercial preparation are available for this use, progesterone and altrenogest are commonly used progestagens in mares. Depending on preparation and vehicle, products will vary of application via (intramuscular, oral or intravaginal implants) and frequency (daily, weekly or every fourteen days). The main differences among the three different via of administration is the residual effect, intramuscular applications show longer residual effects when compared to oral altrenogest or progesterone intravaginal implants, that show a sharp decrease in its effects right after 24 hours of administration [8] or implant removal [19], respectively.

Androgens are steroid hormones responsible to developing and maintaining male characteristics. They are precursor to all estrogens and even though there are many other metabolic active androgens, testosterone is the primary one. In the testis, testosterone is produced by Leydig cells and show endocrine and paracrine actions on male reproduction.

Testicular steroid hormones feedback on the hypothalamus and pituitary to modulate the synthesis and secretion of GnRH, LH and FSH. In the stallion, high concentrations of estrogens are detected in the testis together with testosterone produced by Leydig cells. The production of estrogen comes from enzymatic conversion of androgens to estrogens by the aromatase enzyme, and sertoli cells are more active in producing estrogen than Leydig cells [20]. Steroid hormones concentrations are higher in the testis than in peripheral blood circulation. They are strongly involved in spermatogenesis (paracrine action) and play a crucial role in the hypothalamo-pituitary-gonadal axis function (endocrine action). Estradiol seems to be an important LH surge inductor in the stallion while LH induces a rise up in testosterone concentrations and testosterone controls LH secretion by a negative feedback on the pituitary gland [21].

Anabolic steroids are a class of drugs derived from the testosterone that may be used in the injured horse to rebuild tissues, for build-

ing weight and muscle mass. However, owners and horse couches commonly use these drugs to prepare animals to expositions and to increasing athletic capacity, especially in young horses. The use not associated to a medical purpose may be highly discouraged because of the many side effects it may be causing in the male, and, probably, in the female as well.

Anabolic steroids act as the testosterone in the organism causing a temporally or permanent dysfunction on the hipothalamo-pituitary-gonadal axis, which may lead to a testicular degeneration [21]. The development of a more severe problem and its reversibility will depend on doses, frequencies and period of treatment. Young horses are more sensitive to side effects and pre pubic animals may have an irreversible hipothalamo-pituitary-gonadal axis dysfunction because of its immaturity during treatment [22]. Only two anabolic steroids are approved by U.S. Food and Drug Administration (FDA) for horses, stanazolol and boldenon undecylenate.

Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a fatty acid derivate (eicosanoids) produced from the metabolism of arachidonic acid that is rapid inactivated. In the mare, $PGF_{2\alpha}$ is secreted from endometrium on days 14-16 post ovulation ending diestrus [1,5]. Prostaglandins are involved in a range of reproductive functions and $PGF_{2\alpha}$ actions, promoting luteolysis and myometrial contractions, are widely explored in the reproductive routine.

Natural $PGF_{2\alpha}$ or its analogue is the most frequently administered hormones in the routine. Commercial products may provide a natural $PGF_{2\alpha}$, dinoprost tromethamine, or a synthetic analogue, cloprostenol. Recommended luteolysis doses for dinoprost tromethamine and cloprostenol are 5 to 10 mg and 250 µg, respectively.

Natural $PGF_{2\alpha}$ or its analogues have short half-life but strong size effects when bolus injections are administered. Administrations may

be always by intramuscular via and size effects involve abdominal discomfort and sweating that lasts for a short period of time.

Gonadotropins

Pituitary gonadotropins are glycoproteins composed of two subunits: α and β. The α subunit is common to gonadotropins types and animal species while β subunits is specie and gonadotropin specific. The two pituitary gonadotropins, FSH and LH, are synthesized in the adenohypophisis after GnRH stimulation. Steroid hormones also control gonadotropins synthesis and secretion, estrogens negative feedback on FSH and positive feedback on LH, while progesterone positive feedback on FSH and negative feedback on LH.

Pregnant mares also secret the equine chorionic gonadotropin (eCG) by endometrial calices formed by the chorionic tissue. In the past, eCG was considered to have both FSH and LH function over the pregnant mare ovary, however, it is now more acceptable that eCG shows stronger LH function that is responsible for the luteinization or ovulation of growing follicles in the ovary so that supplementary CL may be formed [8].

The therapeutic or superovulatory (SOV) use of gonadotropins in mares are limited because of the absence of commercial products that are effective for mares. Commercial products include porcine FSH (FSHp) and eCG, which are widely used in ruminants to induce SOV stimulation or the return to cyclicity, however, mares do not respond to these hormones for the same purposes.

Best SOV mares responses have been achieved with equine pituitary extract (EPE) [23], purified equine FSH (FSHe) [24] and recombinant equine FSH (FSHre) [25], however, none of them are available nowadays for a commercial use.

The only gonadotropin commercially available and widely used in mares and stallions is the human chorionic gonadotropin (hCG) because of its LH function. In mares, hCG is used intravenously to

induce ovulation or oocyte maturation [26] and suggested doses vary from 750 UI to 2500 UI [27]. In stallions, because of LH surge induces testosterone rise and influences spermatogenesis, hCG is used to induce testis descent in the cryptorchidism treatment.

Owing to its high molecular weight (36.5 kDa) and glycoprotein properties, hCG induces antibodies formation after repeated injections [28,29]. Controversial results have shown the efficiency of hCG on ovulation induction after repeated oestrous cycles. Some authors have indicated an hCG efficacy reduction after repeated treatments [29-31] while others not [32-34]. The presence of hCG antibodies seems to be deleterious to induce follicle ovulation and oocyte maturation for oocyte collection programs as well, however probably not until the fifth treatment [35]. Siddiqui and colleagues [36] showed that hCG antibodies prevent hCG to increase follicle wall blood flow, to lower oestradiol and free IGF-1 and to raise progesterone concentrations in the follicular fluid, to lower oestradiol and raise LH concentrations in the plasma, and to increase oocyte maturity and quality.

Our more recent options of gonadotropins are the genetically cloned recombinant equine gonadotropins, reFSH and reLH [37-40]. Previous studies have demonstrated its effectiveness on simulating ciclicity in mares after follicular growth suppression (Jennings et al., 2009), induction of multiple pre-ovulatory follicles [40] and induction of ovulation [38]. However, these cloned recombinant equine gonadotropins are not commercial available yet.

Gonadotropin Release Hormone Analogues

Gonadotropin release hormone (GnRH) is a peptidic hormone produced by the hypothalamus with the important function of inducing gonadotropins secretion from the pituitary. It shows a pulsatile release pattern that is essential to define weather FSH or LH is secreted, also, a tonic or pre-ovulatory surge of LH is defined by the hypothalamus centers and GnRH pulsatile frequency.

GnRH analogues are commonly used to induce ovulation in mares and other species females because their injections induce endogenous pre-ovulatory LH increase. There is different GnRH analogues available for commercial use, such as gonadorelin, buserelin and deslorelin acetates, however only deslorelin is effective for mares because of the LH pre-ovulatory rise pattern for this specie. Deslorelin dose for ovulation induction is 1.5 mg injected via intramuscular.

Other recent use for deslorelin in mares is the induction of double ovulations when low doses are used twice a day (BID), but treatment may not be lengthened for more than four days to avoid down-regulation of GnRH receptors [41].

Oxytocin

Oxytocin is a neuropeptide synthesized from neurophysin in the supraoptic nucleus of the hypothalamus and is transported in small vesicles down the hypothalamic-hypophyseal nerve axons.

Oxytocin has a number of functions in reproduction, most of them related to its contractility function over myometrium (uterine contractions) and myoepithelial cells that surround the alveoli in the mammary gland (milk letdown). Another important function is that endometrial concentrations of oxytocin receptor determine uterine prostaglandin F2α secretion in cyclic mares [42], which shows its interference in luteolysis. To control this function during pregnancy, early pregnancy involved mechanisms alter oxytocin receptors function rather than concentration [42].

Oxytocin is routinely used during assisted reproduction programs to explore its myometrium contractility function. Its via of administration influences on the timing of response. Intravenously injection shows faster response compared to intramuscular injection. On the other hand, even though contractility response is dose dependent, doses higher than 25 IU may cause tetanic contractions. For this reason, recommended doses range between 5 IU [43] and 25 IU [44,45] and induce high amplitude uterine contractions for until 30 to

45 minutes [46-49]. Another consideration about oxytocin treatment is the day of treatment, because high progesterone concentrations reduces uterine oxytocin sensitivity, higher doses (20 - 25 IU) may be considered during post ovulatory periods treatments [47].

Dopamine Antagonist

Seasonal reproductive patterns phenomenon is in part regulated by dopamine receptors as evidenced by the presence of synapses between dopaminergic and GnRH neurons in the median eminence and that the inhibition of dopamine receptors was found to be effective in increasing LH secretion during anestrous [50]. Also, dopamine concentration in the cerebrospinal fluid is higher during the anovulatory period compared to the breeding season [51] and it is inversely related to prolactin plasma concentrations [52].

All this knowledge explains why treatment with dopamine antagonist, such as domperidone and sulpiride, has been shown to induce cyclicity in anovulatory mares. It is still unknown by what mechanism dopamine may influences on follicular dynamics, however it is believed that dopamine antagonist could be acting through prolactin at the ovarian level, as prolactin pituitary production is regulated through inhibition by the neurotransmitter dopamine [53].

Hormonal Applications

Ecbolic Function

Uterus contractility is essential for post breeding uterine clearance, parturition and placental membranes expulsion. Failure in any of these processes may lead to a persistent endometritis and reduced reproductive efficiency.

A post breeding mild transient endometritis is expected and a normal response that is controlled by mechanisms of uterine clearance, however, persistent breeding-induced endometritis, where inflammation and intra-uterine fluid retention persist, has a significant

negative impact on fertility. Intra-uterine fluid is one of the more precise signs of endometritis and may be led by a failure in uterine contractility [54], which explains why ecbolic agents are widely used in assisted reproduction programs in mares. The uterine contents are removed in two ways: via lymphatic system and through the cervix and vagina [55], uterine contractions are necessary for both ways [56].

Myometrial contractility is mediated by hormonal (oxytocin and prostaglandins) and neuronal interactions. Prostaglandins and oxytocin increase intracellular calcium concentrations in smooth muscle cells which precipitate formation of actomyosin and initiation of contractions [57]. Oxytocin and prostaglandin F2α, natural and synthetic analogues, such as dinoprost trometamine and cloprostenol respectively, are the routinely used ecbolic agents. However, it is important to consider that mares with delayed uterine clearance show altered patterns of propagation or uterine contractions, reduced number and strength of uterine contractile response, and responded aberrantly to detomidine, a α2-agonist commonly used in fractious mares, and ecbolic drugs. These findings suggest that mares may have an intrinsic contractile defect of the myometrium and possibly a defect in myoelectricial signaling [58] that must be taken into account.

Depending on the breeding moment, post breeding treatment may be done before or after ovulation, which is important to define drugs and doses. Even though progesterone decreases oxytocin uterine contractility, oxytocin is the drug of choice for post ovulation endometritis treatment because PGF2α injection is related to reduced progesterone concentrations and decreased pregnancies rate when used after day zero (day of ovulation).

Of the three prostaglandins analogues evaluated (PGF2α, cloprostenol and fenprostalene), cloprostenol produces the most consistent uterine response [59]. Compared to oxytocin, cloprostenol stimulates weaker but significantly more prolonged uterine contractions (4h versus 30 min) that assist in lymph flow [59,60].

To avoid reduction of fertility by the interference of treatment on sperm transportation, the use of ecbolic drugs should respect drugs half-life (30 min for oxytocin or 4h for cloprostenol) if administration is before breeding, and sperm transport from uterus to oviduct (6h) if administration is after breeding.

Some factors, such as inadequate number of endometrial receptors, a pendulous uterus, a closed cervix and an excessive dose resulting in inappropriate contractions, abnormal propagation of uterine contractions or prolonged inflammation may affect ecbolic drugs function [61]. Some considerations may be highlighted when considering inflammation interference on uterus contractility. First of all, susceptible to post breeding endometritis mares have higher amounts of uterine nitric oxide compared to resistant mares [62]. Nitric oxide show relaxant effects on smooth muscle tissue in general, and it has a dose-dependent inhibitory effect on spontaneous uterine contractility irrespective of the muscle layer in the mare [63]. To increase ecbolic drugs action in an inflammatory condition, uterine flushing may promote a mechanical removal of the lumen free nitric oxide.

Second of all, if a non-steroidal anti-inflammatory (NSAI) drug was used to treat endometritis it is very important to consider its inhibitory effect on prostaglandins actions [64]. Results have suggested that administration of flunixin meglumine [65] or fenilbutazone [61] increases the amount of intra-uterine fluid and the magnitude of the inflammatory reaction. For this reason, these responses may be considered and oxytocin should be the ecbolic drug of choice in mares being treated with NSAI [61].

Retained placenta is an emergency condition that may lead to serious consequences, such as puerperal fever and laminitis. A delay of more than 2 hours until placenta expulsion is already considered retention and the longer the retention, the worse the fertility [66]. Oxytocin is usually used in mares to promote placenta expulsion by intravenous drip infusion containing 30-100 IU of oxytocin and repeated subcutaneous or intramuscular administration of 20-120 IU, but recent works have suggested that intramuscular administration of

50 IU of oxytocin at 1 hour intervals beginning 1 hour after foaling is effective for inducing placental expulsion [67].

Cyclicity Induction

Mares are seasonally polyestrous with regular ovulatory cycles occurring in response to increasing day light. Mare's reproductive activity is divided into breeding season, autumn transition, anestrous and spring transition depending on ovarian follicular growth and ovulation [68]. These variations in ovarian activity along the year is physiological and expected, however, because some horse breed associations determinate that the breeding season may start early in the year there is a pressure to anticipate mare cyclicity.

Acyclic mares may be in deep anestrous or spring transition when mechanisms of cyclicity anticipation may be applied [19,69,70]. Phases are distinguished by follicle number and diameter. Anestrous is characterized by low follicular activity when the largest follicle never exceeds 21 mm (mean diameter, 16 mm) [68]. Mares with at least two follicles of a minimum of 21 mm of diameter that do not achieve dominance characterize the transition period start [71].

The spring transition period has a variable length ranging from about 30 to 90 days and the level of follicular growth can be quite variable among different transitional mares between the early and late transition [68]. Transition ends with the first ovulation of the year and some mares may show around three anovulatory follicles that reach pre-ovulatory sizes every 9 to 10 days until ovulations happen [72,73].

Ovarian activity during anovulatory season is due to hormonal changes, however, even though follicle growth occurs in response to FSH surges during this period, no changes were detected in the magnitude of the wave-stimulating FSH surges in association with an increase in follicle activity within waves during the spring transition [68,74]. The cessation and re-initiation of the ovulatory period are closely associated with the cessation and re-initiation, respectively, of surges in circulating LH [74,75].

Several methods for anticipating mare cyclicity have been tested, including the use of hormones such as GnRH or its analogues [69,76-78]; oral [79,80], intravaginal [70] or injected [81] progesterone; eFSH administration [81,82]; and dopamine antagonistis, such as domperidone or sulpiride [83-85]. Results vary considerably among methods, and the management of the mares during treatments may be an important barrier to their applicability since most of them involve at least once daily administration. Recent studies [69,86] have shown the efficiency of the use of a GnRH pump to accelerate the breeding season establishment, however, because of the need of minor surgery to apply these pumps, the application of treatment in this manner is likely to limit its routine usefulness in the breeding industry.

The more useful method to anticipate cyclicity seems to be progestagens applications. Progestagens are often used to manage the transition period in mares because of its suppression over GnRH pulses frequency that inhibits pre-ovulatory LH surge and consequently LH rise after its progestagen source removal [81]. However, an adequate storage of LH seems to be needed, which is expected in late transition. Variation in efficacy of progestagen treatments is most likely due to the stage of transition at the onset of treatment. In that regard, progestagen treatments do not reliably advance the first ovulation of the year when they commences in anestrus or early transition [81,87,88].

As it has already been discussed, the main difference among the three via of progestagens administration is the residual effect of systemic applications, especially when an intramuscular injection is used. After the intramuscular injection, progesterone shows a rise and establishment of its levels for its half-life followed by a gradually decrease in its concentrations [unpublished data]. On the other hand, intravaginal progesterone-releasing devices show a sharp decrease in its effects right after its removal, promoting better responses in ovulation [89].

So, the use of intravaginal progesterone-releasing devices may be effective in inducing estrus and ovulation in late transition mares few

days after the devices removal [19,70,89], when they are maintained for 10 [70,89] to 12 days [19]. The only reported side effects of intravaginal devices are vaginitis and discomfort [89], but most of mares take it easily during the treatment period.

Because the use of progestagen as an advance of the breeding season in mares is limited to the transition period it seems reasonable to associate it with another method that shorten deep anestrus, such as the use of artificial light. In a study conducted in our research group [unpublished data] in Unesp campus Botucatu, it was possible to observe that artificial light beginning at the winter solstice anticipated transition beginning compared to control group, and the association of artificial light and the insertion of an intravaginal progesterone device when transition was detected anticipated the formation of pre-ovulatory follicle compared to mares only exposed to artificial light.

Once follicle development is initiated and a pre-ovulatory sized follicle is present in the ovary it is reasonable to associate these strategies with an ovulatory induction hormone, such as hCG [69], to accelerate first ovulation.

Oestrus Cycle Manipulation

Manipulation of the oestrus cycle is very important for assisted reproduction because it allows oestrus anticipation with diestrus shortening, a more precise time of ovulation that is based on ovulation induction, multiple ovulations induction and mares synchronization.

Among all hormones, luteolytic agents (cloprostenol and dinoprost trometamine) are the most used in the reproductive routine. With the decrease in progesterone concentrations luteolysis removes the negative feedback on LH which allow a growing follicle to ovulate. So, even though progesterone concentration drops to less than 1ng/mL in 24 hours, the development of oestrus behavior with the presence of a pre-ovulatory follicle depends on the diameter of the largest growing follicle at the moment of luteolytic agent administration and may regard the follicular daily growing rate of 3mm [26]. Using this

knowledge, shortening diestrus is important to anticipate breeding and mares synchronization.

The anticipation may be even more pronounced if the mare is into the 30% that show two major follicular waves (secondary and primary waves) [26]. The presence of a dominant follicle from a secondary wave at the moment luteolytic agent is injected allows oestrus detection on the following day.

The absence of a functional CL with the presence of a dominant follicle determines the onset of oestrus. As soon oestrus is established it remains for few days until ovulation is detected. Oestrus is characterized by receptivity to a stallion, cervical relaxation, presence of a dominant follicle and endometrial edema. The duration of behavioral oestrus averages from 5 to 7 days, but it can vary widely among mares [26]. These difficulties in determining the length of mare's oestrus and the exact ovulation moment are what make the use of ovulation inducing hormones a key tool in the breeding management of mares.

Ovulation Induction

Pregnancy rates are maximized when natural mating is done 48h before ovulation, artificial insemination (AI) with cooled shipped semen is done 12-24h before ovulation and AI with frozen-thawed semen <12h before ovulation to <6h after ovulation [90]. So, because pregnancy rates are associated to breeding at times relative to ovulation, the use of ovulation inducing hormones aims to ensure that only one natural mating or AI is necessary to the establishment of pregnancy.

Besides the need of only one service per cycle, having ovulation at a predictable time include other advantages such as: scheduled breedings, reduced uterine contamination, and ensuring adequate intervals between natural services for specific stallions [90]. Pharmacological agents to induce ovulation include deslorrelin, a GnRH analogue [33]; and hCG [27,91] and reLH [37,38], two gonadotropin hormones. These agents are maximally effective when given to mares with endometrial edema and a follicle ≥ 35 mm in diameter.

Studies comparing different doses of hCG have shown that 750 iu of hCG is effective in advancing ovulation as 1500 iu (both with 92% of mares ovulated within 48h) [27], but even though the small dose may be an alternatively low cost option, 750 iu of hCG did not show a significant increased risk of multiple ovulations compared to not treated mares [27], as seen in previous studies when higher dose rates of hCG were tested, and 1500 iu [92] and 5000 iu [93] accounted for the increase in multiple ovulation rate.

When hCG is used, 75% of induced ovulations occurred between 24 and 48h after treatment [91], while when 1.5 mg of deslorelin in a liquid form is injected intramuscularly ovulations occurred 40-46h after treatment [90]. A recent report also demonstrated that almost 94% of mares treated with 1.25mg of deslorelin injected when a follicle ≥30mm and uterine edema pattern where detected ovulated within 48h and is as effective as treatment with ovuplant [94].

The liquid form of deslorelin for intramuscular injection seems to be a better choice than ovuplant. Ovuplant induced ovulation predictably between 38 and 42h after treatment [95]. However, in some mares that failed to achieve pregnancy, there was a delayed return to the next natural oestrus [96]. In that regard, a small percentage of mares induced to ovulate with Ovuplant had a delay of several days or weeks in returning to oestrus [97].

Together with hCG and deslorelin, reLH would be a much less antigenic option than hCG that showed similar ovulations rates when 0.75 or 0.9mg of reLH was injected. And even though it was concluded that reLH was a reliable and effective ovulatory agent that did not significantly alter endogenous hormone profiles or affect interovulatory intervals [38], it is not commercially available which prevents its routine use.

Multiple Ovulations Induction / Superovulation

Enhanced stimulation of multiple dominant follicles and ovulations with exogenous gonadotropins is generally referred to as follicular superstimulation/superovulation (SOV) in horses [98], even

though the number of stimulated dominant follicles and ovulations is typically less in mares than in cows. This may be the reason of why it is also been known as multiple ovulations induction in mares. Various gonadotropin preparations have been tested, such as crude equine pituitary extract (EPE), purified equine FSH (eFSH®, Bioniche Animal Health, Athens GA, USA), recombinant equine FSH (EquiPure-FSH™, AspenBio Pharma, Castle Rock, CO, USA), purified porcine FSH (Folltropin®-V, Bioniche Animal Health, Athens GA, USA), and recombinant human FSH (Puregon®, Organon B.V., Oss, The Netherlands) administered at different doses, routes, and times during the estrous cycle as well as the spring transitional period.

Even though inconsistent results and the enormous variety of outcomes were seen in the use of different gonadotropins, best results were achieved with EPE [23] and eFSH [25] treatments, and later, reFSH appeared with new perspectives [25]. Many challenges involve SOV in mares and the commercial availability of the preparations is crucial to drive to what is the best protocol to use. Difficulties to purchase gonadotropin preparations conducted to studies with a GnRH analogue called deslorelin that is conventionally available to induce ovulation in mares. Deslorelin showed to be effective to induce double ovulation in 86% from a total of 112 cycles of 56 mares when 100 μg i.m. twice a day from the moment at least two follicles of 20-25mm were detected until both follicles reached 33mm, when ovulation was induced with 2500 iu of hCG [41]. Authors do not indicate lengthen treatment beyond four days because no follicular growth was detected after it in three mares. Follicles then regressed and no ovulation was seen. As seen in other species and suggested by Irvine and Alexander (1993) [99], continuous administration of large doses of GnRH analogues induces an initial hyper-secretion of gonadotropins that is followed to a desensitization of the pituitary gland, promoting a reduction in the release of gonadotropin (downregulation). To ensure that a four day treatment is over at the same moment 33 mm follicles are present in the ovary that the beginning of treatment is set when follicles are 20-25 mm.

There are some important points that involve costs and responsiveness to consider when choosing a SOV treatment: (1) moment to start treatment; (2) hormone dose; (3) injections frequency; (4) ovulation induction. Choosing the best moment to start a SOV treatment involves responsiveness and cost.

Monovulatory species, such as the equine, show a physiological mechanism of selection of the dominant follicle, which will continue to develop even after remarkable follicular environmental changes (high estrogen and high inhibin concentrations decrease FSH), while other follicles from the same follicular wave regress because low FSH concentration is not sufficient to sustain their development. The main target of a SOV protocol is to maintain adequate FSH concentrations for smaller follicles, besides the larger one, to keep developing and capable of ovulating.

To ensure that the start of treatment will be established in a moment where as many follicles as possible are responsive to FSH stimulation, which is characteristic of the common-growth phase, the onset of treatment may occur before divergence of follicles into dominant and subordinates, which occurs when the larger follicle achieves 22-23 mm, as treatment is not able to reestablish growth once follicles begin to regress. When costs, labor and management are considered, SOV treatment should be started right before dominant follicle selection, which means when the largest follicle is 23 mm. Therefore, reduction in days of treatment is achieved with no results impairment.

Not only is the largest follicle diameter important for the onset of treatment but also the diameter of other follicles as well. Even though treatment will stimulate all growing follicles development, when differences among follicles are beyond 5 mm it is not possible to induce ovulation of all of them at once. This is why it is indicated that at least two follicles do not differ more than 5 mm in their diameters.

Another important point to be considered is the luteolysis induction as SOV treatment usually begins during diestrous (common-growth phase). A prostaglandin $F_{2\alpha}$ and its analogues injection

promotes reduction of P4 concentration to similar to what is found during the estrous phase and thereafter removes its negative feedback on LH. Therefore, it is highly recommended that luteolysis may be induced at the beginning of SOV treatment.

Hormone doses and frequencies of injections are important to maintain an adequate bioavailability and level of hormones [23]. Most protocols indicate twice a day injections to mimic a natural hormonal profile circulation. On the other hand, even though higher hormone doses will often promote a hyper stimulation of large numbers of follicles and a high number of luteinic formations, this response may not represent an increasing in embryo recovery. Ovarian hyper stimulation may lead to follicular luteinization without previous ovulation or ovulation with low embryo recovery when more than four ovulations occur in each ovary [100] which may be explained by the presence of an ovulatory fossa that restrain all ovulations to occur at the same place. The formation of blood cloth, edema and all other ovulatory features may justify an ovulatory fossa closure which prevents oocyte pick up by the uterine tube. In summary, doses may be enough to stimulate multiple ovulations (mean of 2-3 ovulations) but not too high to promote ovarian hyper stimulation.

Lastly, ovulation induction is just as important as any other procedures. Ovulation induction is what will able synchronic ovulations and will allow the recover of more than one embryo from a single uterine flushing. Ovulations may be induced with deslorelin or hCG but hCG seems to be a more effective ovulation-induction agent than injectable deslorelin in SOV mares [101].

In Table 1 some SOV protocols described by authors are shown to facilitate practitioners' choice.

Table 1: Summary of SOV protocols and its results using some hormonal preparation already tested.

Hormonal Preparation	Dose	Frequency	Start of Treatment	Ovulation / Cycle	Embryo Recovery*	Reference
EPE	25 mg	BID	D5	4.7±0.6	2.1±0.6 (43.2%)	Scoggin et al., 2002 [23]
eFSH	12.5 mg	BID	D5-7	5.2±0.9	1.6±0.4 (30.8%)	
reFSH	0.65 mg	BID	22-25 mm follicles was present	3.02±0.58	2.0±0.53 (66%)	Meyers-Brown et al., 2011 [25]
reFSH / reLH	0.65 mg / 0.75 mg	BID	22-25 mm follicles was present	4.62±0.88	3.87±0.87 (83%)	Meyers-Brown et al., 2011 [25]
GnRH	100 µg	BID	Largest follicle = 25 mm	1.82±0.5	1.12 (61%)	Nagão et al., 2012 [41]

* Embryo recovery / cycle (% embryo recovery / ovulation)

Mares Synchronization and Recipient Mare Preparation

Mare synchronization is crucial to any embryo transfer or oocyte transfer programs. To know how to choose and prepare a recipient mare is what will lead to good results. The importance of the uterine environment to embryonic development has been highlighted since 1933 [102] and further studies have demonstrated the importance of uterine-embryo synchrony and the effects of the early uterine environment on the subsequent development of the embryo [103]. However, even though a large number of equine embryos are produced and transferred worldwide, the literature is particularly scarce con-

cerning the onset of synchronization protocols of an embryo recipient mare and a donor mare during different stages of the estrous cycle. As a consequence, many protocols preparations, especially for the acyclic recipient mares, are based on empirical evidences.

Synchronization protocols may involve the use of luteolytic and ovulation induction agents for cyclic recipient mares and the use of steroid hormones (estrogens and progestagens) especially to prepare an acyclic recipient mare. It is important to consider how pregnancy recognition occurs in the mare to understand what day of the cycle the recipient mare may be at the embryo transfer moment. Mare pregnancy recognition is achieved by embryo mobility and its signaling to inhibit uterus secretion of prostaglandin F2α, so, to establish a gestation from transferring an embryo to a non-pregnant uterus it is important to give time to this process to happen.

It is recommended that cyclic recipient mares are 5 (58.5 % pregnancy rate on D12), 6 (72.5%), 7 (71.6%), 8 (65.2%) or 9 (59.1%) days after ovulation at embryo transfer moment [104]. Even though many veterinarians are used to associate this preparation with other hormonal (progestagen application) or anti-inflamatory treatments there are no physiologic need that justify them. Progestagen applications may be used to prepare cyclic recipient mares when they haven't achieved the expected days for transfer (D5 - D9) as a way to anticipate an adequate uterine environment to receive the embryo and keep the pregnancy.

The use of steroidal hormones, estrogen followed to long-action (LA) progestagens, are frequently used to prepare acyclic recipient mares in large-scale embryo transfer programs [104 – 110] with similar results (55.6% pregnancy rate) to cyclic mares (67.8%) [104]. The steroidal hormones use is a very important tool to practitioners as a major limiting factor in ET programs is the reduced number and quality of recipient mares during the breeding season, especially the spring' transitional phase.

After recipient uterine preparation with estrogens followed by progestagen treatment, progestagens supplementation is currently administered until 100 – 120 days of gestation in non-cyclic mares, to ensure appropriate progestagen production by the placenta [110]. However, considering the supplementary CL development, it was proposed that exogenous progestagens treatment can be interrupted earlier during pregnancy. That is what was proved by Silva and collaborators in 2014 [8], in an experiment done by our research group. The authors suggest that progestagen treatment interruption in acyclic mares at 70 days of gestation enables it maintenance since supplementary CL are at least five-day-old. These results provide new options to reduce costs and mare management.

A recent study [109] tested the efficacy of prostaglandin, estrogen and progestagen treatment to synchronize acyclic and cyclic recipient to donor mares. The use of steroidal hormones to prepare cyclic recipient mares had not been reported until 2018 [109]. At treatment initiation cyclic mares received one dose of dinoprost and E2, thereafter E2 was repeated on the next three days and LA-progestagen treatment started one day after the last E2 injection. The authors demonstrated that no differences among mares treated during spring transition, early estrous, diestrous and early diestrous (when two doses of dinoprost were given) were detected and satisfactory pregnancy rates ≥65% were reported. Repeating dinoprost to the mares in early diestrous ensured proper luteolysis and response to estrogen as determined by higher uterine edema scores and higher pregnancy rates when compared to the group mares in early diestrous were only given one dose of dinoprost.

Doses of steroidal hormones used for uterine preparation of acyclic, and recently, cyclic mares (depending on the uterine synchronization), are very conflicting. It is well known that concentration of exogenous progestagens is the primary requirement for pregnancy establishment and maintenance in acyclic recipient mares 112]. The demonstration estradiol increases the expression of progesterone uterine receptors and that the equine embryo secretes estrogens dur-

ing the early gestational phase [7] introduced this hormone to most of the acyclic recipient mare preparations.

While plasma progesterone concentration after injection of 1500 mg of LA P4 at seven days intervals has been proved to achieve progesterone concentrations compatible with cyclic mare diestrous [10], there had not been any study with estrogens administrations to compare its concentration after infection in acyclic mares and the physiologic endogenous concentrations during the estrous cycle until few years ago. This led to a wide variability of estrogens dosages that have been used in ET programs what may be found from different studies that used since a single dose of 2.5 mg of EB [8-10] until much larger doses like as seen in a recent report that used three repeated BE administration of 10 mg, 20 mg and 10 mg, respectively [109]. Considering that recipient mares are responsible for carrying a pregnancy to term, it is important to provide an appropriate uterine environment for the conceptus development in acyclic recipient mares.

For this reason, studies were published during the following years, 2016 and 2017 [9,10], in order to test estrogen doses that would induce uterine changes similar to the ones found in cyclic mares. In 2016 [10], the administration of a single dose of 2.5 mg of EB to anestrous mares produced similar estrogen concentration to that found in cyclic mares, while in 2017 [9], the tested protocol (single dose of 2.5 mg of EB followed by 1500 mg of LA P4) produced similar endometrial edema, uterine tonus and changes in relative abundance of progesterone (PR) and estrogen (ERα and ERβ) receptors transcripts to those observed in cyclic mares during late estrous and early diestrous, as well as similar estradiol and estrogen conjugate plasma concentrations. It is important to emphasize that all mares used in the reported studies were not previously treated to steroidal hormones, what might have been influenced with no residual effect.

My personal experience demonstrate that a single small dose such as 2.5 mg of EB is sufficient to induce high uterine edema in

acyclic mares not previously prepared with injectable progestagens and to ensure good pregnancy rate after ET (70%) [unpublished data]. However ideal doses and frequency of injections have not been well established yet. To share more of my experience, thinking about the physiologic hormonal modifications and uterine changes previous to the natural embryo entrance to uterus on D5 – D6, it is possible to prepare mares to receive an embryo by ET before five days after ovulation if a progestagen is given soon enough to induce these uterine modifications. Since 1987 this possibility has been shown when 20 embryos were transferred non-surgically into recipient mares which had been given 22 mg altrenogest daily starting the day of recipient ovulation and higher pregnancy rates (50 vs 0%) were obtained in mares which were 2-6 days after ovulation at the time of transfer compared with mares which were 7-12 days after ovulation [111]. It is possible that not only circulating progesterone concentration is necessary to ensure the establishment of pregnancy but also for how long this hormone has been acting in the uterus to induce uterine environment modifications as well. So, when for some reason I do not have available D5 - D9 cyclic recipient mares for ET, I currently prepare cyclic mares that showed physiologic estrous uterine edema with exogenous progestagens (oral or intramuscular; altrenogest or progesterone) beginning before or together with ovulation, depending on the day of ET, making sure uterus will have at least four days of progestagens exposure.

For the oocyte transfer, the same thoughts must be followed, however, as the oocyte is to be transferred and not the embryo, recipient mares need to be in oestrus. When cyclic recipients are used, they must be synchronized with the donor, and bothe mares receive hCG on the same day. Recipient´s oocyte (s) is collected by follicular aspirations from the dominant follicle (s) to avoid its fertilization. Non-cyclic recipient mares (anestrus or transitional or follicle-suppressed mares) must receive estradiol injections before insemination and oocyte transfer, and them followed by progesterone after transfer [112].

Final Considerations

There are a wide variety of exogenous hormones applications in horse reproduction, and many others to be still developed. Hormonal uses unable veterinarians to manipulate estrous cycle, physiologic events and reproductive biotechnologies appliance. On the other hand, it is necessary to understand hormones characteristics, actions and interactions, as well as the reproduction physiology to ensure an increase in reproductive rates. Protocols do not function alone or solve all problems when an individual treatment is necessary, such as required in horse reproduction. All these possibilities are what make hormonal applications and the veterinarian knowledge the most used tools that enable good results in horse reproduction practice.

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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.

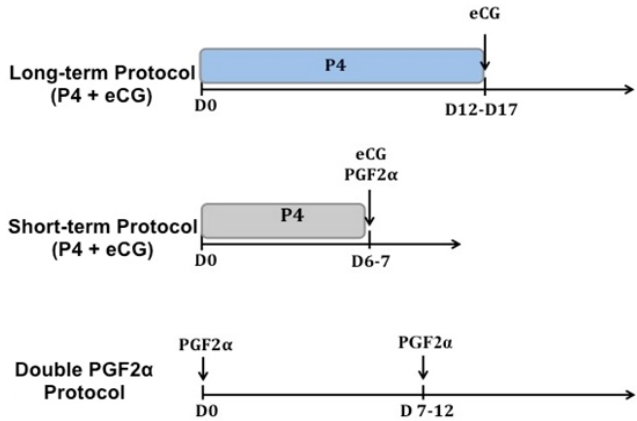

Figure 1: Hormonal protocols used for estrus synchronization in small ruminants.

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).

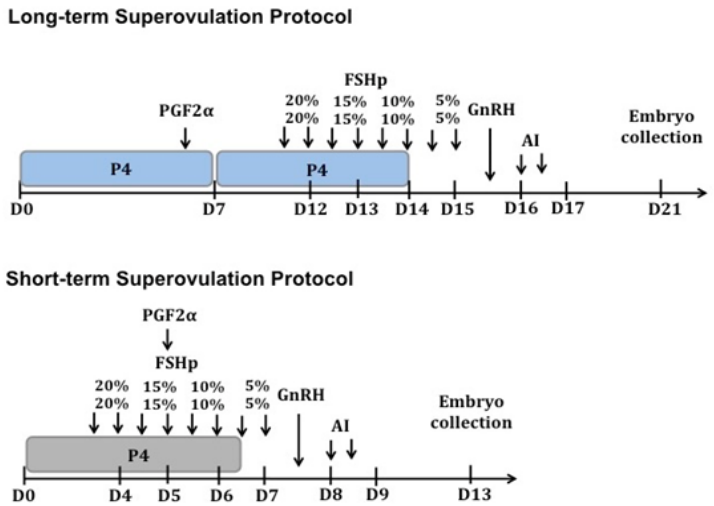

Figure 2: Long- and short-term superovulation protocols commonly used in programs for the collection of embryos in small ruminants.

The ovarian follicular condition, at the beginning of the overstimulation 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).

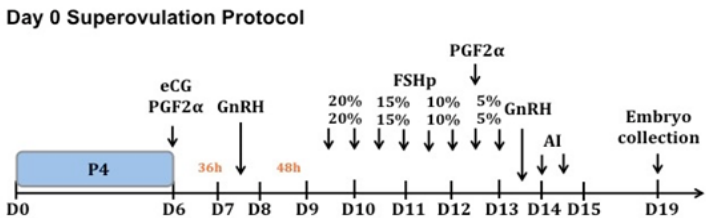

Figure 3: Day 0 superovulation protocol, adapted from Menchaca et al. 2007.

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).

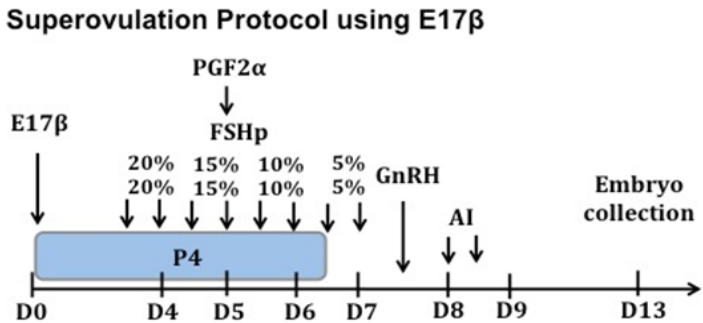

Figure 4: Superovulation protocol using 17β-estradiol for the synchronization of the follicular wave emergence at the beginning of the administration of FSH.

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.

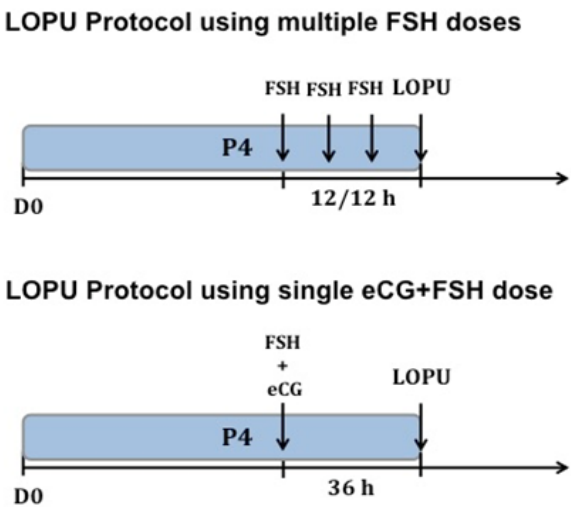

Figure 5: Hormonal protocols for follicular aspiration.

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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Chapter 06

Biotechnics Applied to Bovine Female

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Abstract

Artificial Insemination is an important tool for genetic improvement in cattle with the use of superior genetic material in a large number of herds and at long distances. The possibility of elect a female among many others, on a farm, based on its productive and reproductive history and taking into account their linear classification for type, has driven superovulation. Embryo Transfer is a technique that allows accelerates animal genetic improvement through a selection of donor cows that can have their genetic material multiplied by superovulation and then transferred to selected healthy recipients. It's closely associated with the bovine reproduction process. The recovery of immature oocytes is necessary for In Vitro embryo production.That can be obtained from females through the technique of *ovum pick up.*

Artificial Insemination in Cattle

Artificial insemination (A. I.) has been widely used in dairy herds, mainly due to the support of cooperatives and dairy industries, state and municipal governments, and the entrepreneurial vision of producers. It is an important tool for genetic improvement in cattle because it allows the use of superior genetic material in a large number of herds and at long distances.

In beef herds is effectively used, especially in elite herds, allowing more genetic advances and greater sanitary security. In dairy cattle it's already agreed. In Brazil, the sector still has much to expand. Artificial insemination allows producers to use proven bulls of the best lineages available in Brazil and other countries, through progeny tests, which use information such as:

1) Dairy production averages for milk, fat, protein, total solids and somatic cell counts; information of the conformation for type, such as: height, depth, strength, angularity, udder texture, anterior udder insertion, posterior udder height and width, ceilings length, croup angle, bone quality and calving easy, mainly, for dairy cattle.

2) Birth weight of progeny, weight at weaning, weight development, maternal ability of daughters, racial characterization, precocity and carcass yield, mainly for beef cattle.

In small dairy farms, the non-existence of the bull allows the maintenance of more cows in the herd in production.Sanitary aspects are related to diseases transmitted by natural mating, such as brucellosis, campylobacteriosis, trichomoniasis and vibriosis.With regard to the benefits of A. I., we can list: promotion of genetic improvement for meat and milk; correction of characteristics for type; production of F1 for industrial crossings, and of synthetic breeds; use of bulls indicated for primiparous heifers; elimination of bulls from farms, reduction of expenses and accidents; zootechnical control of the herd and traceability; standardization of the herd; optimizes the use of synthetic breeds with high genetic value; opportunity for producers to work with more than one line of bulls, if necessary; prevents consanguinity.A careful and strict animal and laboratory sanitary control is necessary, as well as efficient breeding programs for the selection of superior breeding herds for the characteristics of interest and free of hereditary diseases.

Conditions for Artificial Insemination

In order for A. I. implantation to succeed in an assisted farm, some conditions are necessary, under the risk of not achieving the expected results, especially in relation to the number of doses used / conception. The farm must have appropriate installations to contain the cow, and trained personnel to detect cows in heat. The inseminator should be empowered, honest and responsible, to have exact control of the stock of semen from the canister. The cylinder must have continuous supply of liquid nitrogen, under the risk of losing all semen stock. Nutrition must be adequate to the productive characteristics of the herd, to the various categories and to the climatic season; this factor is limiting [1,2].

Heat Detection

The correct detection of heat is essential, and requires specific responsibility, skill and knowledge on the part of the observer. This

should present routine of at least two daily observations of 30 minutes each. If ruffian is used, it is recommended one for every 30 cows. It is important that it has libido, dominance and be large. The marker halter can be used with ink. In beef cattle, during the rodeo one must observe calmly, in order not to stress the cows, and not to compromise the observations. The heat is the period in which the female accepts be mated [3].

Limited Failures in the use of A.I

Some factors may contribute negatively to the success of an insemination program, including: failure to detect estrus (poorly trained staff); AI after ovulation (technical failure); postpartum body condition; reproductive pathologies; erroneous conservation of semen; inseminator malpractice; management failures, as regards sanity, food and mineralization [1].

Installations

The basic installations are: C*ontainment trunk for cattle:* preferably a covered trunk, and allowing the cow to be quiet and well contained at the time of insemination, to avoid injury, facilitating the inseminator's work and the examinations of the veterinarian; *Office:* should be near the trunk, where the material used is stored. Also, there must have good quality water for sanitizing the female and equipment.

Bookkeeping

To be economical, animal exploitation needs to be well organized. The control of the herd is facilitated by the use of individual records, where the maximum information about each animal should be recorded. These informations are of real importance to the veterinarian, assisting him in diagnosis and providing him with evaluations of the fertile power of the semen in use and the fertility rates of the herd.

Hygiene

Attention to hygienic practices is one of the most important factors in any process, especially since it is an intra-uterine procedure. On the other hand, in the case of natural mating this does not occur, since the semen is deposited in the vagina, and the possible contamination is prevented by the cervix [1].

Aspects of the Female Reproductive System in Bovine

The first anatomical part of this system is the vulva, which lies just below the anus. In the lower part of the vulva is the clitoris, which is a structure with the function of, when rubbed, to transmit nerve impulses, causing contractions throughout the system, participating in the transport of the semen. In continuity, the vagina is presented, which in the natural mating has the function of receiving the ejaculate. The uterine cervix is a thicker-walled tube, easily identified as consisting of three or four cartilaginous rings, which connects the body of the uterus to the vagina. The uterus is the organ where the development takes place, being responsible for the protection and nutrition of the animal in formation. It can be divided into uterine body and uterine horns (right and left). The uterine tubes are tubular structures of small caliber that connect the ovaries to the uterus. It is in the fallopian tubes that fertilization occurs. The ovaries are two glands where the formation of reproductive cells occurs and also the production of hormones related to the estrous cycle [4].

Artificial Insemination Technique

Recognition of the Signs of Estrus

The estrus is characterized by a series of transformations in the behavior and physiology of the female, caused by the increase in the circulation of estrogenic hormones. It is the phase in which the female accepts the mating.

Approximately 70% of A. I. failure is due to errors in the correct identification of estrus. External signs of estrus: the cow becomes restless and separated from the others; urine frequently, and presents the

vulva swollen and hyperemic, and seeks to keep the tail erect; there is a decrease in milk production and in food intake too; at times, assumes aggressive attitudes; rides on other cows; shows clear and transparent vaginal discharge; the most important sign is when it is allowed to ride by the ruffian or other cows [5].

The inseminator can affirm that the cow is in excellent conditions to be inseminated when observing that it accepts the mating. In healthy and well-nourished cows, estrus occurs at 21 day intervals (ranging from 17 to 24 days), lasting approximately 18 hours in the european breeds. In zebu breeds it is shorter. Normally, 6 to 12 hours after the termination of signs of estrus, ovulation occurs. In zebu breeds, this period can be up to 18 hours [6].

Time for Artificial Insemination

The best time to inseminate is when the exteriorized signs of heat have ceased: the cow can no longer be mated. Due to the difficulty of determining this moment, the following rule is adopted: cows that present heat in the morning, until noon, are inseminated in the afternoon, until 17 hours; cows that are in heat late in the afternoon are inseminated early in the morning. This strategy is known as the Trimberger scheme [1-3,7].

Care in Performing the Insemination

In the office, watch the cow's records, and then in the trunk carry out the correct containment, and clean the animal's rectum. Observe if it's a clear and transparent mucus expulsion. Observe the mucosa of the vulva, which must be hyperemic.

Then perform the hygiene of the cow's posterior region (anus and vulva) by washing and wiping with disposable paper towel. When washing, care should be taken, using the back of the hand, so that there is no opening the vulva. This recommendation is due to the fact that water is spermicidal. Be careful to not contaminate the inside of the vulva. Prepare a blade, paper towel and water at 35ºC. Exteriorize the end of the sheath through a small opening in the plastic wrap on the end side where the applicator should penetrate. Prepare it by

checking the end to be used and remove the metal plunger from the inside by putting it to the side. This will prevent the metal plunger from pushing the vial bushing in advance.

Remove the semen from the canister quickly, thus preserving the other doses that are stored. Always keep the mug up to 7 cm below the mouth of the can. Remove the dose of semen with a clamp in up to 5 seconds. Defrosting should be carried out with the aid of a container containing water heated at 35ºC, remaining for 20 seconds (thin pallets) and for 30 seconds (medium vanes). Dip the vane with the end of the cotton facing down (the water level should completely cover the vane). Dry the reed with paper towel and cut with the blade at the opposite end of the cotton. In the thin reeds the cut should be straight, and the middle reeds should be beveled.

Gently press the plastic plunger out of the sheath with one hand, and engage the cut end of the blade until it snaps into place. Throughout this procedure, the vane should be protected from sunlight. Insert the applicator into the scabbard by pushing the pick up to the tip. Secure the sheath on the applicator by pressing on the plastic ring. Note that the universal applicator has ends of different diameters. Engage the metal plunger by slowly inserting it as far as the vane bushing is located. After inserting the glove, go to the cow, with the applicator properly mounted, and with the proper care of hygiene.

Sequence of Artificial Insemination

Remove the vulvar lips, introduce the applicator assembly at a 45º angle to prevent entry into the urinary meatus. When reaching the bottom of the vagina with the applicator, insert the left hand in the rectum, fixing the cervix. It is sought to orient the applicator towards the hole of the cervix, and carefully, it is tried to dress the cervix in the applicator. You should make movements with the hand that fixes the cervix, and not with the hand that holds the applicator. After transposing the last ring, deposit the semen calmly. The semen should not be deposited in the horn, since ovulation can occur in the right or left ovary. Then the semen should be deposited in the cervix after the last ring [2].

Recommendations

It is recommended to massage the clitoris of the cow after depositing the semen; if it is not successful in passing the third ring, deposit the semen where the end of the applicator is, avoiding lesions; sheaths, gloves, etc., should be conveniently discarded; systematically check the nitrogen level in the bottle, with the appropriate ruler, not allowing it to be below 15 cm, under the risk of loss of all stored doses; note all reproductive events in the binder: dystocic births, miscarriages, stillbirths, retained placenta, etc .; after insemination, write down all the data contained in the reed on the cow records.

Superovulation

The possibility of elect a female among many others, on a farm, based on its productive and reproductive history and taking into account their linear classification for type, has driven superovulation and embryo transfer. Thus, a skilled cow that leaves, for example, approximately six descendants in their useful life, can have a possibility of multiplying that number by eight. For this reason they are modern techniques of animal genetic improvement.

Many factors can influence the final results of a superovulation program, observing the variability of the responses of donor females. Among them: environmental temperature, nutrition, cow age, race, body condition, as well as factors related to the protocol utilized [6,8].

Superovulation makes it possible to neutralize the effect of follicular dominance, and thus the small follicles that would come to atrophy have the possibility of being stimulated, into to ovulate. In normal ovulations, the dominant follicle promotes the reduction of the endogenous concentration of follicle stimulating hormone (FSH) or other gonadotrophin with similar effect; however with the superovulatory treatment this episode is neutralized [9]. Knowing that a heifer contains thousands of follicles in its ovaries, but only a few of them would develop during its reproductive life, superovulation presents a benefit of high relevance. The response to stimulation is related to the

number of follicles present in the ovaries and the stage of the follicular growth wave at the time the superovulatory treatment is initiated [8]. It can also be observed that there is a correlation between the number of follicles present in the ovaries on the day of the initiation of superovulation, which are potentially able to develop, and the number that will ovulate. The follicular population, both at the beginning and during the superovulation, has been shown to be a factor of great importance in the variation of the individual response to the superovulatory treatment [10]. Its use as an evaluation parameter was only possible with the development and use of the ultrasound technique in the semiological approach of the bovine genital system.

There are several proposed superovulation protocols for FSH based products. However, all should consider the stage in which the follicular wave of growth is found at the beginning of treatment. Better results occur when superovulation begins at the beginning of the growth wave. Translating this to the days of the cycle, in animals with two waves per cycle, treatment should begin around day 2 (first wave), or around day 10 (second wave). Eight administrations are generally used, at intervals of 12 hours. Some authors use equal doses, but most prefer decreasing doses. A total dose is calculated and divided into eight smaller doses, in descending order. This scheme can be used for any FSH based product. Prostaglandin is applied simultaneously to the 7th and / or 8th dose of FSH, and aims to regress the corpus luteum formed on day 0 (estrus base), leading to a reduction in the progesterone rate, removing the blockage in GnRH secretion, initiating a new cycle . When the LH wave occurs, it will theoretically find a series of follicles under ovulation conditions due to hormonal induction [8-13].

In the most modern protocols of superovulation, the concern with the synchronization of the follicular emergency is observed [12-13]. So it includes the estradiol benzoate and the progesterone device. Thus, there is no need to observe the estrus stage in the donor to initiate a protocol, and provides the veterinarian with the advantage of

planning the collection dates. It is also sought to decrease the doses of FSH, and currently half of the doses are used if we compare with years ago [11]. Thus, there is an improvement in the quality of the embryos, a lower proportion of side effects in the donors, and the cost savings of superovulation. Another factor considered is the induction of ovulation, which contributes to higher embryo quality, better use of semen and a decrease in the incidence of post-collection ovarian cysts. Synchronization of the onset of the new wave is essential for FSH to be applied at the most appropriate time, avoiding variations in results, with new follicular wave emergence in approximately four days. The efficiency of this association has been observed in females of european breeds. On the other hand, zebu heifers recruit more follicles at the beginning of the follicular growth wave than european heifers, suggesting a greater superstimulatory response if use the treatment with gonadotrophins in zebu females [6]. Since there are occurred the maturation of a large number of ovules at an ovulation, they will encountered with sperm that came from the insemination. If fertilization occurs, from the fifth day we can see the morula, and at that age the embryos reach the uterus too. On the seventh day, we observe the blastocyst stage, which presents a blastocele cavity [11]. This is the time to perform an embryo harvest by washing the uterus before the embryo break up. After the rupture of the membrane pellucida it occurs the break of the embryonic mass, and the embryo happens to be denominated hatched blastocyst. Thus, it's possible to realize a morphological evaluation of the vitality and viability of embryos.

Embryo Transfer

It is a technique that allows accelerates animal genetic improvement through a selection of donor cows that can have their genetic material multiplied by superovulation and then transferred to selected healthy recipients.

Embryo transfer is closely associated with the bovine reproduction process, and helps to increase the proliferation of females that responds better to treatment by more than 70%. They also reduce the

interval between generations, since initial harvests are possible in 15-month-old heifers [9].

These two factors contribute to increase genetic progress by 20 to 30% for a criterion such as milk production, by example. Thus, a transfer of embryos proves to be the safest means of exchanging genetic material between farms, regions, countries or continents. Professionals who work in this area must ensure that all procedures are respected, resulting in sanitary quality of the embryos [9].

While animal insemination remains the main mode of reproduction, capable of generating significant genetic progress, embryo transfer is a complementary tool that, when well founded, can increase the speed of genetic progress. That is why it is applicable particularly in the context of collective selection schemes.Generally, 10 embryos per harvest are obtained, of which 6 are considered good to be conserved or transferred. The ensure pregnancy success rates of these are on average 60% for fresh embryos and 50% for frozen embryos.

There are five steps, which must be carefully carried out, for the success of an embryo transfer program:

1. Superovulation

It is induced by injections twice daily for 4 days of preparations containing gonadotropic hormones, essentially FSH, derived from purified extracts from pig hypophysis and with safety quality verified previously. On average, the number of embryos observed after FSH treatments is about 12, but there is a very large individual variation that is still impossible to predict (ranging from 0 to 50 embryos). It is expected that one in five females doesn't have ovulation.

2. Harvesting of Embryos

It is performed on the seventh day after insemination of the donor cow. It is performed through introducing a catheter into the uterus of the female after the passageway of the cervix. This is a delicate technique that requires a lot of skill and experience.

The procedure takes about half an hour. It consists of injecting a liquid buffer into the uterine cavity to rinse and retrieve it, bathing the embryos that are present, and thus, by gravity, withdraw them. The buffer solution used, with pH 7.2 ± 0.2 and controlled osmolarity (300 mm Osm / kg ± 30) maintains the medium under conditions conducive to the maintenance of embryos for short periods of time. Its composition normally have sodium chloride; anhydrous sodium phosphate; glucose; potassium chloride; potassium phosphate; magnesium chloride; sodium pyruvate; calcium chloride dihydrate; amikacin sulfate and ultrapure water. After collection, embryos are searched in the liquid medium by filtration or decantation.

3. Evaluating Embryos Quality

It is essential to evaluate the fertility expectations and ensure the necessary health qualities. The embryos are placed under a magnifying glass and their quality is estimated according to their morphological aspect due the time after insemination and due to the integrity of the pellucid membrane that surround the embryo.

There is an international scale for classification, produced by the International Embryo Transfer Society (IETS), encoding embryo quality from 1 (excellent) until 4 (degenerate or unfertilized oocytes).

4. Wash and Preservation of Embryos

Wash the embryos in 10 successive baths of buffered solution is mandatory. This guarantees an embryo harvest free of pathogenic germs.

These washed embryos are then stored. The time between a harvest and a transfer should not exceed eight hours. In case of freezing in liquid nitrogen, the embryo should not wait more than four hours. In the latter case, the embryo is stored in a medium containing cryoprotectant. A freezing method based on ethylene glycol is used, allowing direct transfer of the embryo to a recipient after thawing. This method, currently the most widespread, does not require embryo manipulation.

5. Transfer of Embryos

The embryos are placed individually in the uterus of a female called the recipient, whose estrous cycle was more frequently controlled so as to be perfectly synchronous with that of the donor. Often it is preferred to use heifers to receive the embryos, because that they must be in good health. All receivers must have a careful examination of the reproductive system. They should be regularly in estral cycle and weigh at least 2/3 of the weight of an adult cow. In the case of adult cows are utilized as receivers, these should have passed the puerperium period being between 50 and 80 days postpartum.

For direct transfer, the recipient cows should be as close as possible to the female donor. Seven-day-old embryos should be implanted in cows who have presented estrus for seven days ago. It is an essential factor in successful transplantation.

Synchronization should be performed according to traditional methods of inducing estrus, such as the placement and removal of progesterone implants. Early detection of cycles for each animal should be accurate to eliminate any poorly synchronized animals. For each donor subjected to superovulation, it is advisable to prepare six to eight heifers as receivers. Unused animals may be inseminated in the following heats.

At the time of transfer, each recipient is evaluated. It is then determined which ovary contains the corpus luteum, in order to deposit the embryo in the corresponding horn. The embryo is transferred using an applicator similar to that used for insemination. The cervical route is used, which is similar to insemination, and therefore an experienced professional is necessary. It is recommended to perform pregnancy diagnosis by rectal examination after 30 days. A heifer that exhibit heat shows that the process has failed, and can be inseminated normally.

Oocyte Recovery and *In Vitro* Embryo Production

Oocyte recovery and in vitro production (IVP) techniques are directly related, due to the dependence of being used together.

Oocyte Recovery

The recovery of immature oocytes is necessary for embryos IVP. That can be obtained from females in vivo through the technique of *ovum pick up* (OPU) or in vitro ovaries of postmortem females. Regardless of the technique, oocytes are obtained between 3-8 mm in follicular diameter [14-15].

In OPU, oocytes are obtained through ultrasound-guided follicular aspiration. A needle attached to a vacuum pump is used transvaginally to perform aspiration of the follicle through ultrasound imaging [16]. In the in vitro technique, oocytes are obtained from ovaries of slaughtered females, punctured with the aid of a needle attached to a syringe.

Throughout the process, regardless of the technique, the temperature should be controlled at 35°C to maintain the oocyte viability for the IVP of embryos. Aspects such as race, age and donor nutrition should be considered for the success of the technique, since low quality oocytes reflect in reducing the rates of embryo production [17-18]. Differences in the number of oocytes recovered between donors are expected [19].

Oocyte Classification

The recovered oocytes are evaluated by cell morphology [20]. Homogenity of cytoplasm, presence of cumulus cells, cumulus compaction and brown staining are aspects evaluated (Figure 1 and 2).

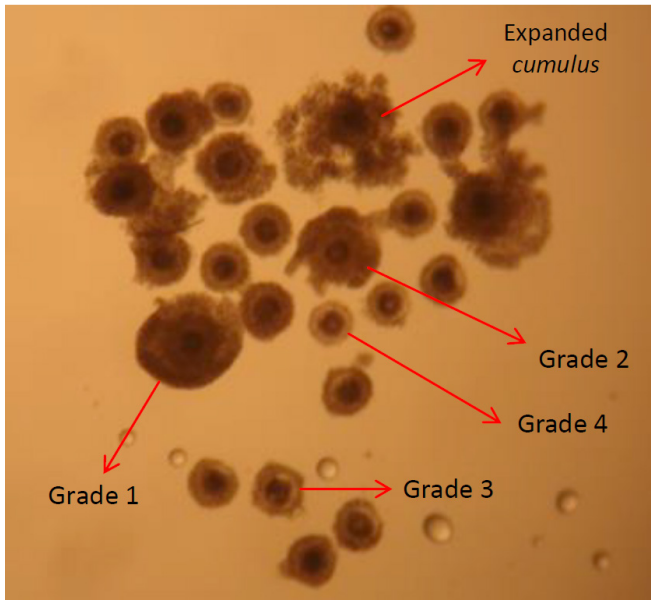

Figure 1: Oocyte classification by morphological appearance.

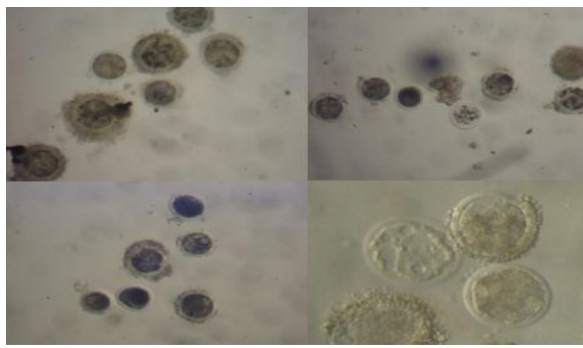

Figure 2: Oocytes with heterogeneous cytoplasm, vacuolated and without cumulus cells, obtained from Holstein females at peak lactation, raised in a tropical climate region.

Grade 1: Cumulus-oocyte complex (COCs) with homogeneous cytoplasm, cumulus cells completely surrounding the zona pellucida (ZP), compacted and with multiple layers.

Grade 2: COCs with homogeneous cytoplasm, areas of irregular pigmentation, cumulus smaller than grade 1, but with more than five layers of packed cumulus cells completely surrounding the ZP.

Grade 3: COCs with heterogenous, vacuolated cytoplasm with three to five layers of cumulus cells surrounding ZP, except for small areas without cumulus cover.

Grade 4: COCs with heterogeneous, pigmented cytoplasm with totally or largely absent or expanded cumulus.

In Vitro Production of Bovine Embryos

In The IVP of embryos is a technique that consists of the preparation of gametes and embryonic development in vitro. The IVP allows to obtain the largest number of progenies of the same female among all reproductive biotechniques.

In the acceleration of genetic improvement, the IVP allows a producer to obtain in a generation a herd of high racial or pure origin with the purchase of gametes from pure animals of origin.

The greater number of progeny also allows a greater pressure of selection and reduction of the interval between generations, using females of high productive and racial potential as donors of oocytes for the production of embryos and females of low potential or without racial pattern as recipients of embryos IVP.

The greatest advantage of IVP is the flexibility to be used in herds of any fitness and size, to produce commercial animals for slaughter or milk production, or to produce elite animals, breeding animals and matrices.

Currently, Brazil is the world leader in the bovine embryo IVP, with more than 350,000 bovine embryos in 2015 and represents 57% of the world production of embryos in vitro [21].

The technique consists of three basic steps: In Vitro Maturation (IVM) of oocytes, in vitro fertilization (IVF) of oocytes and in vitro culture (IVC) of embryos.

In Vitro Maturation of Oocytes

Immature oocytes (Figure 3) go through the IVM process. In IVM, the oocytes undergo a cellular reorganization that corresponds to nuclear and cytoplasmic maturation.

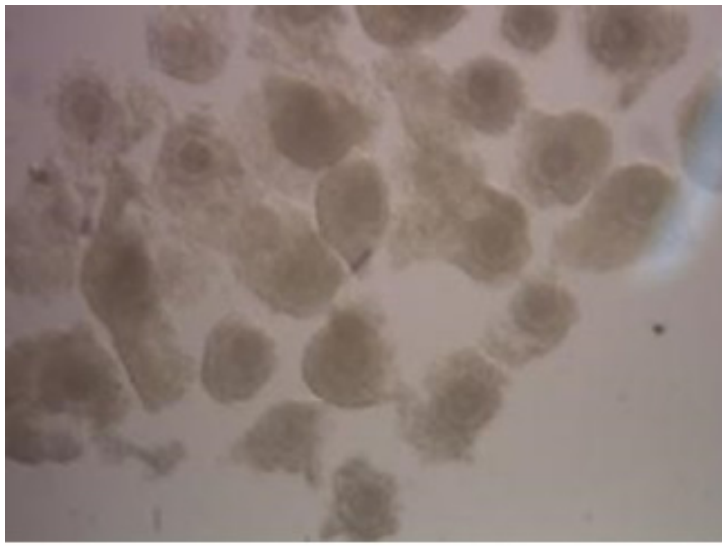

Figure 3: Aspect of immature oocytes recovered by OPU.

Nuclear maturation consists of the resumption and progression of meiosis to the metaphase stage II (MII) and the extrusion of the first polar corpuscle. Cytoplasmic maturation is a process of difficult evaluation, consisting of the reorganization of cytoplasmic organelles and mRNA synthesis [14,22].

The occurrence of the two maturation processes allows the oocyte to have potential for embryo development when fertilized, and the production of blastocysts is a reliable factor for indirect evaluation of oocyte maturation [15,22].

The occurrence of IVM begins spontaneously with the withdrawal of the oocyte from the follicular fluid. For the structural and biochemical events of maturation to occur, it is necessary to meet the nutritional and physico-chemical requirements of the cells [23].

The IVM medium consists of TCM 199 containing glutamine, fetal bovine serum (FBS), pyruvate, follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol and amikacin sulfate. Oocytes are matured in vitro for 22-24 hours at 38.7°C (Figure 4) [23-28].

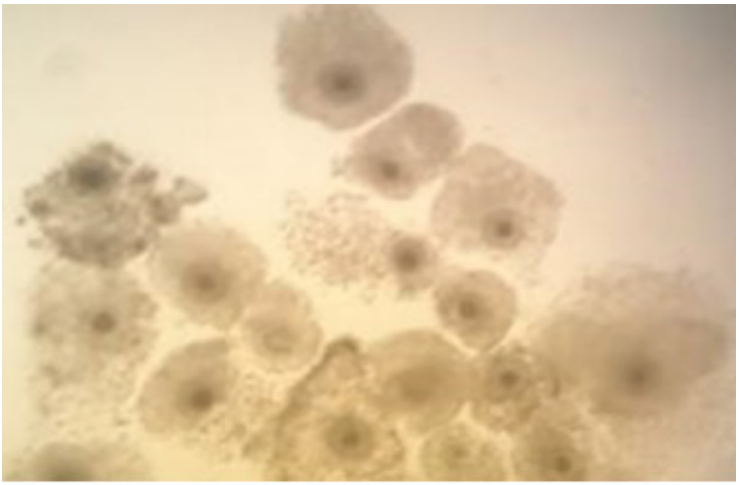

Figure 4: Appearance of oocytes matured after IVM.

In Vitro Fertilization of Oocytes

After IVM, the oocytes will be assigned to the IVF stage, where fertilization will occur. For fertilization to occur spermatozoa should be selected. Sperm selection is performed using two methods: Percoll® gradient or Swim up [14-15].

In the Percoll® gradient the spermatozoa are selected by centrifugation, and the living and dead spermatozoa are separated by the colloidal silica particles at different positions of the gradient.

In the Swim up method, the semen is deposited in a test tube and covered by a small amount of culture medium. The best spermatozoa with rectilinear motility swim to the surface. After a period of 30 to 60 minutes, the supernatant is removed, which contains the spermatozoids that have been able to reach the surface.In both methods spermatozoa with rectilinear and progressive motility are desired, due to the capacity of fertilization [14].

With sperm selection the fertilization dishes are inseminated with the concentration of 1 to 2 x 10^6 sperm per mL. The mature oocytes are placed next to the spermatozoa so that the fertilization process takes place [14-15].

The IVF medium consists of Fert-TALP(tyrode-albumina-lacta-to-piruvato),that is composed by a set of physiological salts, added fatty acid free bovine serum albumin (BSA-FAF), pyruvate, PHE (penicillamine, hypotaurine and epinephrine), heparin and amikacin sulfate [25-28].

The gametes remain bound in IVF for a period of 18 to 22 hours at 38.7°C. During this period, the sperm cells undergo the process of sperm capacitation, gaining motility and vigor by the PHE. Capacitive agents such as heparin will allow greater membrane fluidity by performing the acrosomal reaction and binding the zona pellucida to the occurrence of fertilization. With the occurrence of fertilization the zygotes begin their development [14-15].

In Vitro Culture of Embryos

In the IVC, nutritional conditions are met using CR1, CR2 and SOF, consisting of a set of physiological salts, supplemented with BSA-FAF, SFB, glutamine, alanine, glycine and amikacin sulfate. For a period of seven days of incubation at 38.7°C the zygotes will go through the process of cell cleavage until reaching the stage of blastocyst to

be innovated in the receptors initiating their gestational development [23-28].

During the IVC, zygotes initiate cleavage, reaching the 16-cell stage, where they pass through the activation of the embryonic genome itself (materno-zygotic transition), continuing its development until the blastocyst stage, where they present the first cellular differentiation (trophoblast and embryoblast) [14-15,28-29].

Embryonic Stages

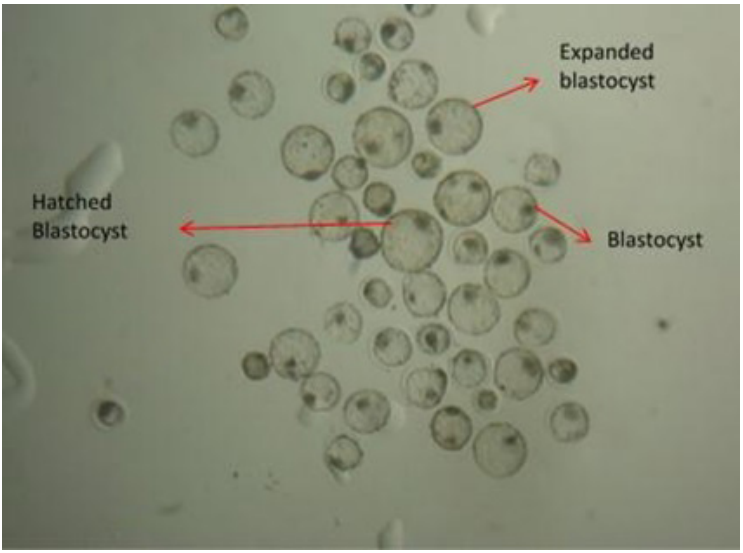

Figure 5: Classification of the embryonic stage.

Morphology of the embryonic stages (Figure 5) evaluated on day 7 (D7) of culture in vitro [14-15,30].

a) Blastocyst - Embryo with first cellular differentiation (trophoblast and internal cell mass), formation of blastocele, presence of embryonic bud, without blastocele expansion, zona pellucida.

b) Expanded blastocyst - Conformation of the blastocyst with expansion of the blastocele and zona pellucida thin, but intact.

c) Blastocyst hatching - Blastocyst conformation, with rupture of the zona pellucida and partial or complete extrusion of the embryo out of the zona pellucida.

Gaseous Incubation Atmosphere in IVP

The *in vitro* culture with atmosphere gas is the balanced composition of CO2, O2 and N2. Two systems are currently used, the open system and the closed system.

The open system is most commonly used in IVP of production animals and the closed system is applied to biotechniques such as nuclear transfer cloning and IVP of human embryos [2].

The open system is where the atmosphere of the cell culture incubator is formed by CO2, O2 and N2, however, only the CO2 level is controlled and maintained at 5% in free air.

In the closed system the CO2, O2 and N2 levels are controlled maintained in the proportion of 5%, 5% and 90%. Throughout the IVP process the moisture must be saturated.

The pH balance is important for the occurrence of the biochemical events of the cell. The pH in culture media of the IVM, IVF and IVC steps ranges from 7.1 to 7.5.

In the IVP the most applied buffer medium is HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*), avoiding fluctuations in the incubation systems.

OPU-PIV Considerations

For the effective success of the technique the objectives of its application must be clear. The application of the technique is effective in the production of animals of high genetic standard due to the ac-

celeration of the annual genetic gain and the possibility of increasing the selection pressure in pure herds of origin.

From the industrial point of view, the technique allows to increase the gains in the production of industrial crossbreeding embryos between breeds, sexed and of high potential of meat or milk production. The use of donors with high oocyte recovery and, especially, good embryo conversion rates is fundamental for the success of the technique. The IVP, more than a technique of genetic multiplication, should be used for genetic improvement and increased production. Insisting on the use of the technique using donors with low oocyte retrieval is frustrating, lacking the appropriate return on investment.

Conclusion

Biotechnologies applied to breeding have been improved to expand the genetic materials of the male and female line in cattle breeding, respectively. So the farmers have the opportunity to expand the best results. Veterinarians responsible for reproductive programs are developing protocols with the objective of improving performance, increasing the birth rate and reducing the interval between generations, obtaining greater productivity taking into account improvements in handling and nutrition of the herds.

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Chapter 7

Artificial Insemination and Embryo Transfer in Small Ruminants

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Abstract

Reproductive biotechnologies are important tools for genetic improvement because they enable to increase the gene frequency of individuals with superior genotype. Among the reproductive biotechnology applied to sheep and goats, artificial insemination (AI) and embryo transfer (ET) stand out. The techniques for AI and ET of sheep and goats differ from those normally used in other farm animals mainly due to their anatomical peculiarities. The methods of IA and ET are described in a simple and practical way in this chapter. The results after AI and ET were reviewed in the literature to establish the expected results of each technique. In conclusion, this chapter reviews the main reproductive biotechnologies applied to ovine and caprine females.

Sheep and Goat Anatomy and Estral Cycle

In sheep, some anatomical peculiarities limit the application of traditional reproductive biotechnology used in cattle. Because of ewe/goat small size, it is not possible to perform rectal palpation to fasten the cervix for the semen applicator or Foley catheter passage into the uterus. Another important factor is that the ewe has the cervix formed by cartilaginous and mismatched rings (Figure 1), making it difficult to access the uterus by transcervical applicators. The number of rings and length of the cervix varies according to sheep breed and age [1].

Sheep estrus cycle has 17 days on average and can vary from 14 to 19 days. Goats have an estrous cycle of 21 days and can vary between 18 and 22 days. The estrous cycle is divided into a follicular phase for two to three days and luteal phase of 14 to 15 days. Small ruminants are reproductively seasonal species. Although the season exerts little effect on the spermatic quality of the male [2], the females are strongly influenced by the year season. The decrease of the photoperiod stimulates the beginning of the breeding season, with sheep and goats presenting several estrous cycles in autumn and winter. During the summer and winter, sheep/goats remain in anestrous. However, seasonality may vary according to the breed and the location. Woolly breeds tend to be more seasonal than the hair breeds [3]. The higher the latitude of the sheep environment the greater the restriction to the seasonal period [4].

Artificial Insemination

Advantages of AI include genetic improvement when semen from superior genotype rams are used; the possibility of using semen from rams not adapted to unfavorable production systems; the reduction of the number of rams required in the production system; the decrease in the effect of seasonality on production when methods of induction or synchronization of estrus are used; the concentration of births and the consequent rationalization of the labor force and the reduction of the interval between births. On the other hand, the disadvantages of AI include the need for specialized labor; the increase of the management in the property and the identification of estrus.

The nomenclature of AI techniques varies according to the location of semen deposition in the female reproductive tract. Figure 1 shows the locations of semen deposition and names of AI techniques in sheep and goats.

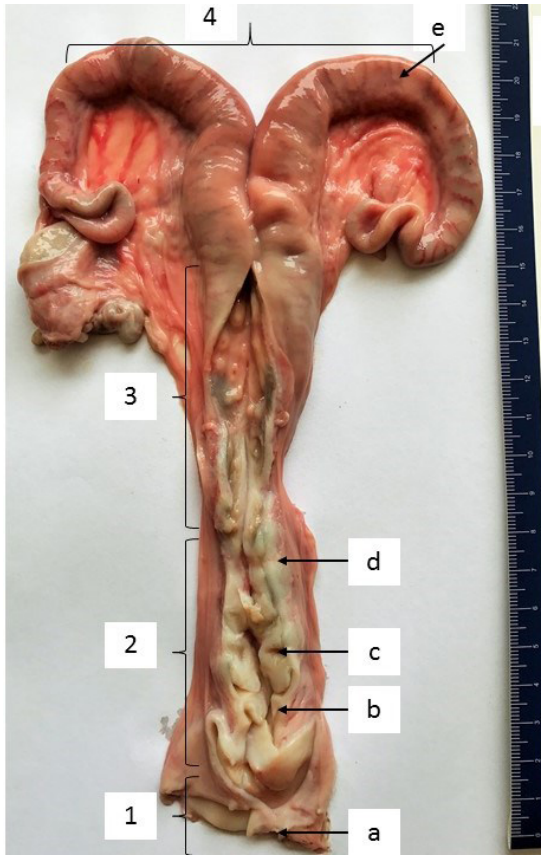

Figure 1: The Reproductive tract of the ewe. 1. Vagina 2. Cervix 3. Body of the uterus 4. Uterine horns. The lowercase letters indicate the site of semen deposition in the various AI techniques (a. Vaginal b. Superficial cervical c. Deep cervical d. Transcervical Laparoscopic).

The deeper in the female reproductive tract the semen is deposited lower the inseminating dose required and higher the chance of fertilization and consequently the higher the rate of gestation.

Artificial Vaginal Insemination

In vaginal insemination technique, the semen is deposited in the vagina, as close as possible to the entrance of the cervix. The ewe should be positioned with the rear elevated at a 45° angle, usually, a trestle or the wall of the restraint is used for positioning. The technician uses a vaginal speculum and a light source to visualize the entrance of the cervix and the semen is deposited as close as possible to the entry of the cervix with the artificial insemination gun.

The inseminating dose for this technique is 200 to 400 x 10^6 spermatozoa and the volume of 0.2mL [5]

Superficial Cervical, Deep Cervical, and Transcervical Insemination

Cervical insemination techniques are the procedures of choice for artificial insemination of goats because of the rectilinear anatomy of the cervix in this specie.

In this technique, the sheep/goat must be stationed. It is advisable to use an elevated shearing table to prevent ergonomic injury to the inseminator technician. The use of sedation in the female is indicated, however after this se-

dation sheep or goat should be able to be stand. After dry cleaning of the vulva and perineum and with the help of speculum vagina and light source, the inseminator must identify the entrance of the cervix and proceed to apprehend the vaginal sac with two atraumatic forceps. Then the assistant should gently pull the tweezers in order to extend and fix the cervix. This apprehension enables for the inseminator to trans pass the cervical rings with the semen applicator.

In cervical insemination, the semen is deposited in the female's cervix. This technique can be classified as superficial when it is possible to pass to three cervical rings or deep when more than four cervical rings are crossed, but the cervix is not completely traversed. The inseminating dose for cervical AI is 100 to 450 x 10^6 spermatozoa [5].

Transcervical artificial insemination is performed when the inseminator succeeds in traversed all cervical rings and the semen is deposited in the body of the uterus. In this technique, the inseminating dose is 0.5 mL with 100 to 450 x 10^6 spermatozoa [5]. This technique allows the use of frozen/thawed semen with satisfactory results.

It is important to emphasize that the proceed of pass the semen applicator must be performed gently to avoid lesions of the vagina or cervix mucosa in order to avoid bleeding that may decrease sperm viability or lead to complications in the female such as vaginitis, cervicitis, fetal pathway stenosis, among others.

Laparoscopic Insemination

In the technique of artificial insemination by laparoscopy, the semen is deposited directly in the uterine horns of the female. The procedure is surgical and should be performed only by a veterinarian.

The ewes or goats must undergo food and water fasting for at least 12 hours. The animals are sedated and placed in dorsal decubitus on barrows with angles of 45° (Trendelenburg position - Figure 2). Depilation and antisepsis of 15 cm cranially to the mammary gland is indicated. The incision of the skin is performed in the ventral region approximately 7 centimeters cranially to the mammary gland. The length of the skin incision depends on the diameter of the trocars used as a portal. After skin incision the trocar with inflation cannula must be inserted into the abdominal cavity, then the abdominal cavity is blown with compressed air or purified CO^2. The procedure of opening the second portal, which will be used to insert the semen applicator, follows the same sequence as the first portal, but with a distance of approximately 8 centimeters from the first one. The second trocar does not need to contain an inflation cannula. The authors suggest that the two trocars for AI are restricted to the left side of the alba line in order to reduce the incidence of adhesions and scars on the right side that can be preserved for eventual laparotomy embryo collection procedures.

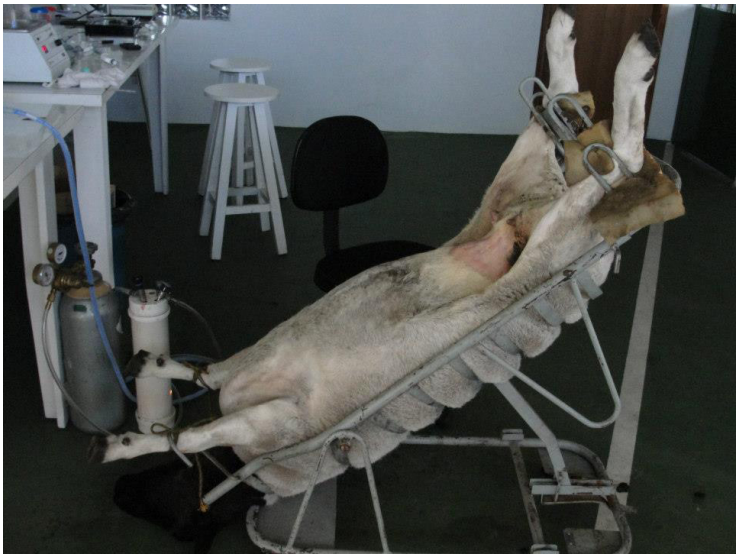

Figure 2: Ewe in Trendelenburg position.

The optic coupled to a light source is inserted into the first portal for visualization of the uterine horns (Figure 3A). After identification of the uterine horns the semen applicator, loaded with the total semen dose of 20 x 10^6 spermatozoa divided into two columns of approximately 0.125 ml, is inserted by the second portal. The inseminator performs the perforation of serosa on the greater curvature of the two uterine horns and deposits a semen column on each uterine horn (Figure 3B). Then the optic and the semen applicator are removed, the portals remain for a few seconds to expel the gas used in the pneumoperitoneum, the portals are removed and the skin incisions are sutured [6,7].

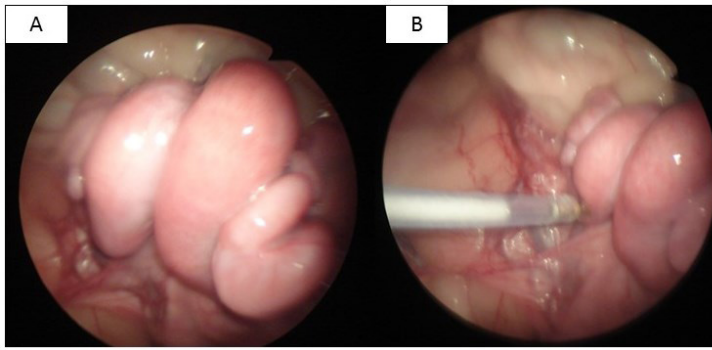

Figure 3: Laparoscopic image of artificial insemination by laparoscopy. A. Uterine horns B. Deposition of semen in the greater curvature of the uterine horn with Robertson's applicator.

AI Results in Sheep

The success rate or rate of gestation of AI varies according to several factors, eg. semen preservation method, synchronization protocol, the season of year, breed, insemination method. Table 1 shows AI results with several methods of preserving semen and AI techniques.

There is a consensus among the authors that the techniques of choice for AI with cryopreserved semen are those that allow intrauterine deposition of spermatozoa, with transcervical and laparoscopic procedures being the most indicated. However, in sheep, the wooly breeds have the conformation of the cervix more tortuous than the hair breeds, making it difficult to transpose the cervical rings with the semen applicator. Casali et al. [13] reported

that the passage of all cervical rings (transcervical IA) was possible in less than 10% of the 586 Corriedale sheep used in the study.

Table 1: Pregnancy rate references after artificial insemination with fresh, refrigerated or frozen semen in the various methods of insemination in sheep.

Reference	Sperm	AI Method	Pregnancy rate
[8]	Frozen	Superficial cervix	32.2%
		Deepcervix	44.4%
		Laparoscopic	57.1%
[9]	Refrigerated	Vaginal	14.8%
		Laparoscopic	77.2%
[10]	Refrigerated	Vaginal	35.7%
		Laparoscopic	71.8%
[11]	Fresh	Vaginal	58.8%
	Frozen	Vaginal	14.7%
	Frozen	Laparoscopic	61.7%
[12]	Frozen	Superficial cervix	16.3%
		Deepcervix	17.1%
		Transcervical	27.2%
[13]	Fresh	Vaginal	36.0%
		Transcevical	42.3%
		Laparoscopic	50.2%

Embryos Transfer

Embryo transfer biotechnology (ET) is applied to females of superior genotype and aims to increase the frequency of their genes by increasing their progeny. ET allows the transference of embryos from superior females (donors) to matrices with low genetic value (recipients) or embryo freezing for later use.

Embryo Transfer Process in Sheep

The stages of ET (Figure 4) consist of the selection of donors, recipients, and reproducers; donors superovulation; recipients ovulation synchronization; AI; embryos collection and embryos destination that can be transferred to recipients or frozen for later use.

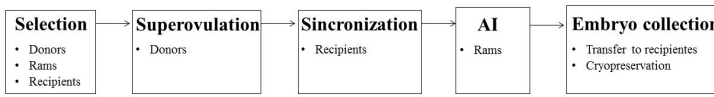

Figure 4: Embryo transfer process.

Donor selection is based primarily on genetic merit. Sheep or goats that have acquired diseases of the locomotive apparatus, mammary gland or others that enables them from carrying the term pregnancy can also be selected. Rams used in AI process should have good sperm quality and superior genotype. Recipients selected for an ET process should be healthy, have a good maternal ability and milk production. Mixed ewes of dairy breeds tend to be optimal recipients.

Donor superovulation is performed with exogenous hormones that inhibit dominant follicle differentiation. Superovulation protocols are based on equine chorionic gonadotrophin(eCG) or follicle stimulating hormone (FSH) after a period of progestogen administration [14]. The authors suggest the following protocol: donors cycles

synchronization using intravaginal devices impregnated with 0.33 g of progesterone (Eazi-Breed CIDR®, Zoetis, USA) for 14 days, the devices should be replaced with new ones on day seven of treatment. Superovulation induction by the administration of 240 mg NIH-FSH-S1 FSH-P1 (Folltropin®, Telesta Therapeutics Inc., Canada) divided into eight applications of decreasing doses from the twelfth day of the treatment. At the time of the last FSH injection, the progesterone implant should be withdrawn and 200 IU eCG (Novormon®, Zoetis, United States) should be administered. The donors remained with vasectomized rams for 56 hours after the withdrawal of the progesterone implant.

Recipients synchronization follows the protocol of donors synchronization with the progestogen, the application of the eCG and the ruffiation. It is important to note that the recipients will be in estrus phase together with the donors, however under no circumstances can these receptors be exposed to non-vasectomized males.

Between 50 and 56 hours after progestogen withdrawal, in vivo fertilization of ovulated oocytes should be performed. Fertilization can be performed by natural mating, but the ram must perform more than one coverage in order to fertilize all the released oocytes. The AI has superior results in fertilization of superovulated sheep/goats [15]. Two AIs with six hours interval between them, in the final third of estrus, should be performed.

The technique of choice for ovine embryos collection is laparotomy. The donor should be fasted for at least 24 hours. The donor is placed in dorsal decubitus in containment table with 45° angulation. Depilation and antisepsis of the ventral region should be performed, approximately 30 cm cranial to the mammary gland. The authors suggest sedation with Acepromazine 1% at the dose of 0.05mg/kg IM and inhalation anesthesia with Isoflurane. The skin incision can be performed on the alba line or 5cm on the right and parallel to the alba line. The incision of the skin, subcutaneous, abdominal musculature and peritoneum should have approximately 7 cm in diameter or sufficient for the introduction of two surgeon's fingers. Then the uterine horns should be externalized, avoiding the exteriorization of the ovaries. A Foley catheter should be positioned in the initial third of each uterine horn (Figure 5B). After the inflation of the probe balloon, the surgeon inserts a 20G catheter into the final third of the uterine horn. Twenty-five milliliters of Dulbecco's modified DMPBS Flush (PBS) is infused from the catheter and collected through the Foley probe into a petri dish. The same procedure should be performed in the contralateral uterine horn. Then the uterine horns should be repositioned and the suture of the musculature (Sultan or Reverd in suture with absorbable thread), subcutaneous (continuous suture with absorbable thread) and skin (separate single stitches with unabsorbable thread) should be performed.

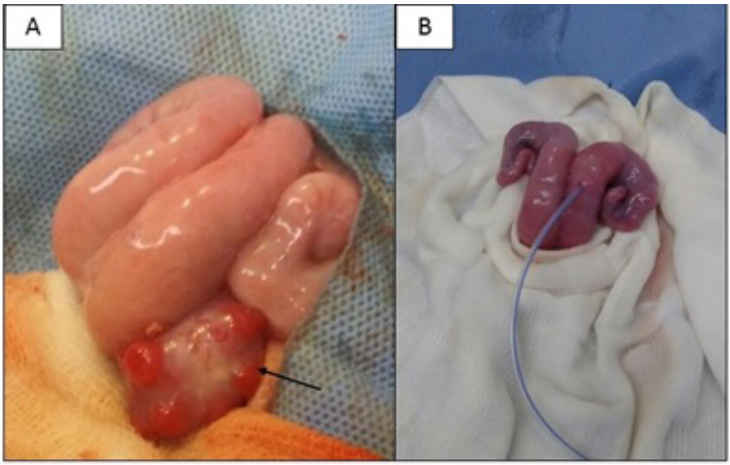

Figure 5: Embryo collection in sheep. A. Positioning of the uterine horns, highlighted ovary with five visible ovulations. B. Positioning of the Foley catheter in the initial third of the uterine horns.

After collection, the petri dish is taken to stereomicroscope to search and classify the embryos collected (Figure 6A). The embryos may be cryopreserved or transferred to the recipient.

Embryos should only be transferred to recipients who have ovulation. After identification of ovulation of the recipient by laparoscopy (Figure 6B), using a Babcock tweezer the final third of the uterine horn with ovulation is externalized and the embryo is implanted.

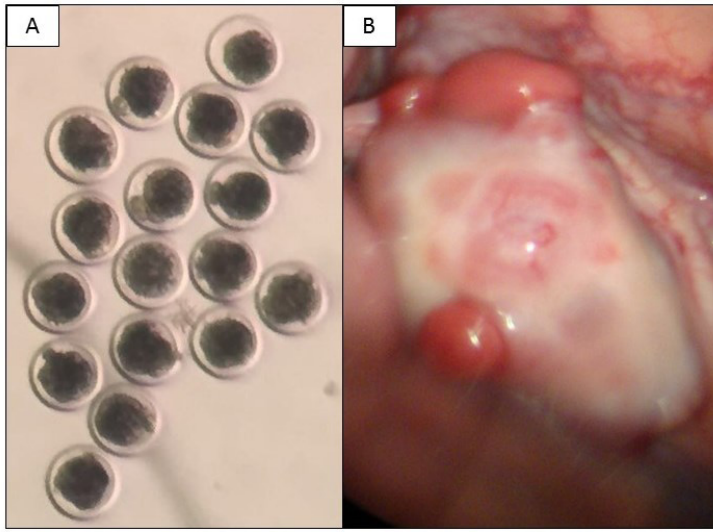

Figure 6: A. Sheep embryos in the morula stage. B. Laparoscopic image of the verification of three corpora lutea in embryo recipient.

Expected ET Results in Sheep

The programs of ET after superovulation present great variability of results in the number of embryos collected and the rate of gestation after transfer. This variability is the result of the individual factors of donors [16], a number of collections performed in the same donor [16], superovulation protocol [15,17], fertilization method [15], the breeding season [18] among others.

However, the average number of embryos collected per ewe is between 4 and 13 embryos and the rate of gestation after transfer between 37% and 80% [15–20].

Conclusions

Reproductive biotechniques of small ruminants differ from those of other species, mainly due to their anatomical peculiarities. Unfortunately, the variability of results is a limiting factor in the application of reproductive biotechnology in sheep and goats. In addition to studies in the field of biotechnology, concern about the correct handling of the estrous cycle, animal welfare, sanitary and nutritional management of animals can reduce the variability of the results making these biotechnologies more widespread.

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Chapter 8

In Vitro Embryo Production in the Pig

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Abstract

Pigs show economic importance and physiological similarities to humans. For this reason, the *in vitro* production (IVP) of swine embryos is an important biotechnological tool. However, despite the advances in IVP of mice and cattle, the progress in this specie is relatively slow. *In vitro* pig embryos are obtained by a series of integrated and efficient manipulations of follicular oocytes. The IVP process includes four technical steps: (1) Oocyte collection (ovaries from slaughterhouse), (2) *In vitro* maturation (IVM) of immature oocytes, (3) *In vitro* fertilization (IVF) of matured oocytes and (4) In vitro culture (IVC) of zygotes. In this chapter we have discussed and provided different steps of IVP and general notions of the physiological processes, as well as recent advances and obstacles of IVP of embryos in pigs.

Introduction

Swine breeding is an important economic activity in the world. Data shows that, in European Union (EU), swine meat represents 9.0 % of the total agricultural output and, from 2009 to 2014, it has increased by 3.6 %, while bovine meat output has decreased by 5.7 %. The production in the European Union was driven by the export surge of swine meat to China, low feed prices and a higher number of breeding cows, reaching 22.9 million tons [1].

Furthermore, pigs show physiological similarities to humans, that has ended up rising the economic interest in swine biotechnologies by both biomedical and swine industries. The interest has created an increased desire for new technologies as well as an urge for implementation of the existing ones [2]. At this point, the *in vitro* production (IVP) of swine embryos is interesting to researchers. These animals can be used as human biomodels and for creating genetically modified animals as potential donors of tissues and organs for xenotransplantation [3].

The first approach using IVP technology in pigs was established in 1986 [4] from *in vitro* fertilization (IVF) of in vivo matured oocytes. After that, in 1989, the first production of piglets by IVP was reported [5]. In that study, *in vitro* matured and *in vitro* fertilized oocytes produced 2-cell to 4-cell embryos after 44 h post-insemination (post insemination - pi). Since then, studies have shown that successful large-scale IVP of porcine embryos can provide viable embryos more efficiently, with less cost and in less time, when compared to the surgical collection of *in vivo* derived embryos from sows. For *in vitro* culture (IVC), procedures have improved, however, for *in vitro* maturation (IVM) and IVF systems still have several unsolved problems, including imbalance of nuclear and cytoplasmic maturation and polyspermy [6].

Porcine *in vitro* embryos are obtained from a series of integrated and efficient techniques by manipulation of follicular oocytes *in vitro*. The process includes four technical steps: (1) oocyte collection (ovaries from slaughterhouse), (2) IVM of immature oocytes, (3) IVF of matured oocytes and (4) IVC of zygotes. According to the currently applied protocols in immature porcine oocytes, there is a lot of variation in the results, but around 20–30% develop to blastocyst stage [7,8]. However, a substantial proportion of embryo polyploidy is a problem observed in porcine IVP [6].

In this chapter we will discuss and provide different steps of IVP and general notions of the physiological processes, as well as recent advances and obstacles observed during *in vitro* maturation, fertilization and culture of pig embryos.

Oocyte Collection and *In Vitro* Maturation

Collection of good-quality oocytes is the first step for *in vitro* embryo production. There are two sources to collect oocytes in pigs: surgical procedures or, more commonly, ovaries from slaughterhouses.

The ovaries, obtained from abattoir, should be transported to the laboratory within 2h, immersed in 0.9% NaCl or phosphate-buffered saline (PBS) solution, supplemented with 75 µg/mL potassium penicillin G and 50 mg/mL streptomycin sulfate, at 30-35^{0}C. Only follicles between 3–6 mm should be aspirated by an 18-gauge needle, attached to a 10-mL disposable syringe, or connected to Falcon tube, under controlled vacuum (30 mm Hg). Then, cumulus oocyte complexes (COCs) with a uniform ooplasm and compact cumulus cell mass should be selected under a stereomicroscope for IVM [9,10].

Before moving to the next step of IVP, attention must be paid to the quality of culture media used. These solutions are of critical importance and will reflect on subsequent embryo development. In that perspective, the improvement of media used for IVM, IVF and IVC brings reliability and reproducibility of results among different laboratories [11]. Another key factor that can affect the success of culture media is the osmolality [12]. It was proposed that the osmolality of IVM, IVF and IVC media should remain stable during incubation [13]. The osmotic stresses can damage DNA and affect DNA replication, DNA transcription and mRNA translation, leading to cellular disturbance [14]. Porcine oocytes cultured with hyperosmotic solutions (>300 mOsm/kg) lose the ability to develop into blastocyst in one-half of oocytes evaluated, showing the importance of osmolality control [15].

Low temperature is another variable that can be harmful to oocytes. Temperature reduction is associated to ruptures of the cytoskeleton as it also decreases cellular metabolism [16,17]. The ideal temperature for culture in pigs is around 38.5^{0}C [18].

After oocyte collection, the next crucial step is IVM. The oocyte maturation involves cell changes that transform an oocyte unable to progress, to a one able to support the events of fertilization and embryo development. During the process, immature COCs undergoes meiosis, reducing their chromosomes quantity to half, extrusion of the first polar body, reorganization of all organelles, to finally receive the spermatozoa [19].

For IVM of pig oocytes, several basic culture medium types have been used, including North Carolina State University (NCSU) medium [20], modified tissue culture medium (TCM 199) and modified Tyrode's medium, containing lactate and pyruvate (mTLP) [21]. These media are generally supplemented with porcine follicular fluid (pFF), or serum. However, pFF is not available commercially and it shows variability in its properties, depending on how it's prepared. In contrast, fetal bovine serum (FBS) is commercially available, with quality assured by the supplier, showing several advantageous components, such as low levels of antibodies and numerous growth factors [22,23]. The oocytes matured in NCSU-37 medium, supplemented with FBS, supported blastocysts to develop into piglets after IVF and IVC [24].

Supplementation of different hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), pregnant mare serum gonadotrophin (PMSG), human chorionic gonadotropin (hCG), estradiol-17β, leptin and relaxin, in IVM medium, has shown beneficial effects on oocyte maturation. Moreover, growth factors and other substances, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), growth differentiation factor 9 (GDF-9), Transforming growth factor (TGF)-α, epidermal growth factor (EGF), EGF-like peptides and L-carnitine, also show improvement of oocyte maturation [22].

A problem usually observed in porcine IVM oocytes is the reduced developmental ability as compared to their *in vivo* matured counterparts, suggesting cytoplasmic insufficiency (Nagai et al., 2006). However, the cytoplasmic maturation of IVM oocytes could be improved by protecting oocytes from oxidative stress, caused by reactive oxygen species (ROS) [25].

Glutathione (GSH) is known to be an important intracellular factor that can control cellular levels of ROS and protect the oocytes against the damaging effects of oxidative stress [26]. Therefore, the ideal IVM medium should be capable to enhance the cytoplasmic GSH contents of matured oocytes. Stimulating synthesis of intracel-

lular GSH is the main beneficial effect of IVM medium supplementation with cysteine and EGF [27,28].

Zinc is an essential trace mineral required for normal fetal growth and development. And also, zinc is known to influence pregnancy, embryonic development, and fetal survival in mammals [29]. The zinc supplementation in IVM medium was recently assessment [30]. The addition of 0.8 μg/mL zinc increased the intracellular GSH concentration, reducing the ROS level, during porcine IVM, with beneficial effect on blastocyst formation.

To achieve an ideal IVM, immature COCs should be incubated in maturation medium, chosen according to protocols, and cultured for around 44 hours, at 38.8 ^{0}C, under an atmosphere air of 5% CO2, with maximum humidity [9].

In Vitro Fertilization

In vitro fertilization is a crucial step in the embryo IVP procedure. However, several variables are involved in this process. For proper IVF, fresh or cryopreserved semen must be prepared before incubation with oocytes. Seminal plasma and/or extender contain components that function as decapacitation factors and must be removed before co-incubation with oocytes [31]. Thus, before sperm capacitation for IVF, boar spermatozoa have conventionally been washed to separate them from seminal plasma and extender by simple centrifugation [32,33]. It appears to resist a high g-force (2400 x g), for a relatively short centrifugation time (3 minutes) [34].

Percoll gradient centrifugation has been used and showed higher *in vitro* penetration rates and increased cleavage and blastocyst formation rates after IVF [35,36,37]. Another method of sperm separation is swim-up. The procedure has been successfully used to isolate a highly motile sperm population [38]. Moreover, beyond separating good sperm for IVF, it has been reported that the modified swim-up method, based on general sperm swim-up technique, could reduce the occurrence of polyspermy in pig oocytes, during IVF. It increases

the outcome of normal karyotypes, leading to an improved development potential in porcine embryos [39].

At the end of pig oocyte maturation, cumulus oocyte-complexes (COCs) should be separated from cumulus cells, in a process called oocyte denudation. The COCs can be denuded by pipetting or vortexing and, regardless the methodology chosen, both procedures result in similar final rates of embryo development. However, researchers found that the denuding procedure should be used with care, concerning: induction of oocyte damage, changes in the position of the first polar body regarding the metaphase spindle, the spindle pattern, and cytoplasmic maturation [10].

For IVF procedure, the oocytes should be incubated with a sperm concentration defined by IVF protocol. Studies using 1 to 4.5 x 10^5 cells/mL showed good results [9,10]. It is important to notice that cell culture incubator at 38.8 °C and a humidified atmosphere of 5% CO2, for a period of 18-24 hours, is the ideal environment to perform IVF.

It is not easy to choose one among all the culture media that have already been tested for IVF in pigs. However, once selected, the basal fertilization medium is always supplemented with different additives to improve the results. It is the selection of these molecules that will designate the nature of the medium: chemically defined or undefined. Thus, there are some options of different media that can be used. Table 1, below, describes the main composition of basal IVF media used for swine culture [revised by 31].

Table 1: Composition of basal IVF media used in the pig.

Component (mM)	mTBM	mTALP	mTCM-199	PGM	PGMtac4
NacL	113.10	114.06	116.35	108.00	108.00
KCl	3.00	3.20	5.36	10.00	10.00
KH_2PO_4	-	-	-	0.35	0.35
$MgCl_2 \bullet 6H_2O$	-	0.50	-	-	-
$MgSO_4$	-	-	0.81	-	-
$MgSO_4 \bullet 7H_2O$	-	-	-	0.40	0.40
Na-lactate 60% syrup. (mL/L)	-	1.85	-	-	-
NaH_2PO_4	-	0.35	1.01	-	-
Glucose	11.00	5.00	3.05	1.00	-
$NaHCO_3$	-	25.07	26.19	25.07	25.00
Caffeine	1.00	2.00	5.00	-	-
Ca-(lactate)$_2$	-	-	2.92	-	-
Ca-(lactate)$_2 \bullet 5H_2O$	-	-	-	2.50	4.00
Tris	20.00	-	-	-	-
Na-pyruvate	5.00	0.11	0.91	0.20	0.20
$CaCl_2 \bullet 2H_2O$	7.50	4.70	1.80	-	-
Sorbitol	-	-	12.00	-	-
Polyvinyl-Alcohol (mg/mL)	-	1.00	-	3.00	3.00
Theophylline	-	-	-	-	2.50
Adenosine (μM)	-	-	-	-	1.00
Cysteine (μM)	-	-	-	-	0.25
Gentamicin (mg/mL)	-	-	-	0.05	0.01
Penicillin G/ streptomycin	-	-	0.17/0.07	-	-
Amilkacin sulfate (mg/mL)	-	0.10	-	-	-
BSA (mg/ml)	1.00	3.00	4.00	-	-
Reference	[32]	[40]	[41]	[42]	[42]

*mTALP: modified Tyrode's albumin lactate pyruvate; mTBM, modified Tris-buffered medium;

mTCM-199, modified tissue culture medium-199; PGM, porcine gamete medium; PGMtac, porcine gamete medium theophylline–adenosine–cysteine; mTCM-199: partial listing of components of TCM-199 with Earle's salts and L-glutamine (cat. no. M-5017; Sigma). There are different supplementations of TCM.

The success of embryo development to the blastocyst stage during IVP relies on oocyte cytoplasm to facilitate normal fertilization and subsequent embryo development [43]. The process by which immature oocytes acquire that ability, during IVM, is frequently called cytoplasmic maturation. High polyspermy rates and low blastocyst developmental rates are considered to be the two major obstacles in porcine IVP [6]. Those obstacles may be related to insufficient cytoplasmic maturation during IVM.

During IVF process, penetration of the oocyte cytoplasm by more than one spermatozoa may occur and is considered a pathologic condition, observed in placental mammals, called polyspermy. Usually, it causes early death of the embryo. Particularly in pigs, this defect affects approximately 50% of *in vitro* fertilized oocytes [44]. Since the release of the first studies about IVM-IVF in pigs, problems of polyspermy and male pronucleus formation were often observed. Researchers thought that polyspermy could be simply resolved by reducing the numbers of sperm available to the mature oocyte, during IVF. However, that approach has been proved wrong and it does not provide a solution [45].

Physiologically, the oocyte fertilization in mammals induces cortical granules (CG) exocytosis, which is initiated by calcium oscillations during sperm penetration [46]. Moreover, it induces the fusion of the CG to the ooplasma and the exocytosis of their content into the perivitelline space, which modifies the oocyte plasma membrane and the zona pellucida (ZP), turning the oocyte refractory to additional sperm binding and penetration [47]. Other mechanisms occurring inside the oviduct, reducing the mass arrival of spermatozoa in the

vicinity of the oocyte, also have been proposed as contributing to the blockage of polyspermy [48].

The final maturation of the oocyte in the oviductal environment is suggested as a necessary step for successful fertilization and embryo development [49,50]. Indeed, many existing studies support a functional role of the oviduct and its secretions (which are rich in estrus-associated glycoproteins) during fertilization. They regulate processes such as sperm–ZP binding, establishment of species-specific ZP barriers and early embryonic development [51,52].

In that perspective, the efficiency of the incorporation of oviduct fluid (OF) in IVF medium have been tested. Recent studies show that the incubation of matured oocytes with pure OF for 30 minutes, before IVF, or 10% OF, during IVF, decreases the number of ZP sperm-bound and the incidence of polyspermy, regardless of sperm concentration [53].

Those findings can be explained by the presence of important contents in OF that contribute to the regulation of fertilization. Several proteins in the OF can bind to the ZP, modifying both its protein and carbohydrate composition [53]. Moreover, hypothesis have been raised stating that the sperm-recognizing labels coming from OF can be incorporated in the ZP, helping the selection of specific subpopulations of capacitated spermatozoa, even though, the final number of spermatozoa around the oocyte, at the fertilization time, is reduced *in vivo* [44].

The presence of OF, before or during IVF, allows to increase the sperm concentration, without affecting the monospermy rate [44]. The exposure of oocytes to pure OF, before IVF, or diluted OF (10%), during IVF, improved the efficiency of production of monospermic zygotes. The most effective combination for achieving a higher monospermic zygote production in IVF is: sperm concentration of 4.5×10^5 cells/mL and oocyte exposure to OF, either before or during IVF [9].

The OF can be obtained by selecting and treating collected genital tracts from slaughterhouses. Briefly, based on ovarian morphology of both ovaries, the oviducts at late follicular phase should be selected by established criteria [54]. Thereafter, these oviducts should be quickly washed, once, with 70% ethanol solution and, twice, with Dulbecco's PBS, and then, transferred to cold Petri dishes (on ice), to be dissected free of surrounding tissues. The oviducts, flushed with 500 mL of PBS, should be inserted into the ampulla tip for washing the lumen. That procedure should be repeated with 10 oviducts. The same fluid recovered in the first oviduct should be reused to wash the next one. Finally, the oviductal flush should be centrifuged at 7000 x g, for 10 minutes, at 4 ^{0}C, to remove cellular debris. Then, the supernatant must be immediately stored at -20 °C, until its use [9].

Another alternative method to obtain less polyspermic fertilization andto achieve higher developmental competence of in vitro-matured oocytes is the use of modulators of cAMP during pig IVM. One of these modulators is dibutyryl cAMP sodium salt (dbcAMP), which has been used during IVM to increase cAMP levels, postpone meiosis, and consequently synchronizes nuclear and cytoplasmic maturation of the oocytes [55,56]. The results, when using that substance, show that the addition of dbcAMP in the medium, during the first 22 hours of IVM, induced a lower penetration rate followed by a lower polyspermy rate after IVF. Ultimately, it results in a higher percentage of fertilized zygotes in pigs [55].

In Vitro Culture

IVC is the last step of embryo IVP in pigs. Revising the entire process, immature COC are obtained from ovaries (1), then, *in vitro* matured (2) and, *in vitro* fertilized (3). The zygotes will undergo cleavage (4), will develop into morulae (5) and, finally, will develop into a blastocyst (6). Figure 1 shows the schematic morphologies of embryo IVP.

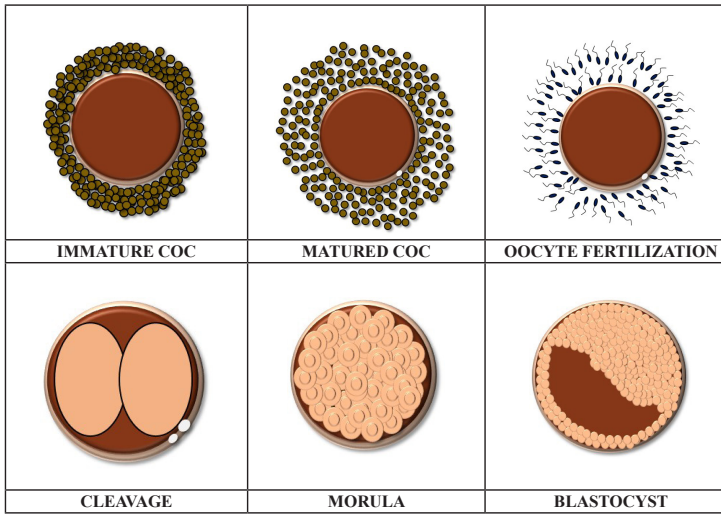

Figure 1: Schematic representation of stages of embryo development in pigs. COC: *cumulus*-oocyte complexes.

IVC conditions are the main variables that can affect quality and yield of blastocyst development [57]. For ideal embryo transfer, the use of high-quality blastocysts (with numerous nuclei and lower apoptotic index) increases the rates of pregnancy success [58]. Thus, the media composition is one critical element to achieve excellent quality blastocysts. Usually, the IVC medium contains defined levels of different energy sources: glucose, calcium, lactate and pyruvate, as well as supplement vitamins [57].

Early-stage embryos are the ideal ones for transfer, especially because it minimizes *in vitro* exposure. However, that would preclude the assessment of embryonic developmental capacity. It was observed that the ideal culture time for porcine embryos is 5 to 6 days. When those embryos are cultured for 7-8 days, they show a lower quality and a higher apoptotic frequency [58,59]. That phenomenon can be

explained by the increase of the ROS (also called free radicals). High levels of ROS cause oxidative stress, decreasing trophectoderm (TE) cells in IVP blastocysts, which results in delayed post-implantation development [60,61].

In that perspective, the use of strategies to avoid increase of ROS must be pursued. Thus, the IVC performed in low oxygen levels (5% of O2, 5% of CO2 and 90% of N2) [62,63,64] may reduce the ROS levels. However, some researchers control only CO2 levels, without controlling oxygen levels (using 5% of CO2 in atmosphere) [65,66].

Media used for IVC can be classified according to their composition and divided into three groups: (1) simple media, originally developed for mouse embryos (for example: Simple Optimized Medium - SOM, Chatoz Ziomec Bavister-CZB); (2) media derived from the liquid composition of the oviduct (for example: Synthetic Oviductal Fluid - SOF, Human Tubal Fluid - HTF); and finally, (3) medium designed to culture of somatic cells (for example: Tissue Culture Medium - TCM199) [67,68].

In swine, the most commonly media used are: simple medium NCSU-23 (North Carolina State University) and oviduct liquid composition medium PZM-3 (Porcine Zygote Medium). The use of PMZ-3 supplement by FBS reducing the ROS levels, which leads to improved blastocyst development and hatching rates [65]. Table 2 shows the composition of these media [reviewed by 63].

Table 2: Composition of IVC media used for pig.

Component	**PZM-3**	**NCSU-23**
NaCl (mM)	108.00	108.73
KCL (mM)	10.00	4.78
$CaCl_2.2H_2O$ (mM)	–	1.70
KH_2PO_4 (mM)	0.35	1.19
$MgSO_4.7H_2O$ (mM)	0.40	1.19
$NaHCO_3$ (mM)	25.07	25.07
Glucose (mM)	–	5.55
Na-Pyruvate (mM)	0.20	–
Ca-$(lactate)_2.5H_2O$ (mM)	2.00	–
L-Glutamine (mM)	1.00	1.00
Taurine (mM)	–	7.00
Hypotaurine (mM)	5.00	5.00
Basal Medium Eagle amino acids	20.00	–
Minimun Essential Medium nonessental amino acids	10.00	–
Gentamicin (mg/ml)	0,05	0.05
Fatty acid-free BSA (mg/ml)	3.00	4.00
Osmolarity (mOsm)	288±2	291±2
Ph	7.3±0.02	7.3±0.02

*NCSU-23: North Carolina State University; PZM-3: Porcine Zygote Medium.

Protocols used in porcine IVC shows variation among laboratories. A big challenge to be outpaced is ensure the reproducibility of protocols, and establishment of blastocyst rates. Table 3 shows the rates of blastocysts obtained by comparing the IVC in different atmospheres and media.

Table 3: Blastocysts rates in different media and atmosphere.

Atmosphere	IVC medium	Blastocysts rates (%)	Reference
5% CO_2	PZM-3	48.9±4.1*	[65]
		35.5±3.2**	[65]
		11.2±0.5	[10]
		61.7	[63]
	NCSU-23	34.2 ± 10.7	[11]
		59.6	[63]
5% O_2, 5% CO_2 and 90% N_2	PZM-3	24.0	[62]
		65.9	[63]
		28.2± 3.3	[30]
	NCSU-23	32.6	[63]
		16.6	[64]

Data are the mean±SEM; Blastocyst rates: number of blastocyst/total of oocytes cultivated.
NCSU-23: North Carolina State University; PZM-3: Porcine Zygote Medium.
*IVC medium supplemented with fetal bovine serum (FBS).
**IVC medium without FBS.

Conclusion

In the present chapter, main steps of embryo IVP in pig were presented to understand the recent advances and barriers. Despite the biotechnological improvements, a high proportion of embryo polyploidy and variations of embryo development among laboratories is observed. However, recent strategies presented, such as, supplementation of oviductal fluid, Zinc, modulators of cAMP and incubation in low oxygen levels can improve the embryo IVP in pigs. Presently, further studies should be implemented to increase the production of good quality embryos capable of developing to term.

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Chapter 09

Equine Semen Biotechnology

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Abstract

The equine industry has been adopting modern biotechnologies that allow the reduction of production costs and selecting breeding animals with superior genetic merit due to the fact that productivity is essential for positive economic results. Among these advances, the biotechnologies of semen are important instruments to maximize the use of stallions, prevent disease spread, eliminate geographical barriers and increase semen longevity. Although essential to accelerate genetic improvement worldwide, cryopreservation of equine semen has not yet achieved the same successful results found in cattle. Therefore, research has focused on developing new semen extenders, processing techniques and methods of insemination in order to enable this biotechnology on a larger scale in the equine species. In this sense, this chapter aims to review the main reproductive biotechnologies related to stallion.

Cooled Semen

Artificial insemination (AI) with cooled semen is the most commonly used biotechnology in the equine, since the results obtained with frozen semen remain unsatisfactory [1,2].

The main purpose of refrigeration during sperm storage is to decrease cellular metabolism, which then reduces toxic product generation from catabolism, thus lessening further damage to cells. The decrease in temperature by 10° C leads to a 50% drop in cellular metabolism, so the sperm maintained at 5° C present 10% of the initial metabolism (38° C), after ejaculation. The metabolism reduction increases sperm longevity [3] with conception rates similar to fresh semen [4,5].

While a decrease in temperature increases sperm longevity, a rapid decrease in temperature can lead to changes in sperm plasma membrane stability. Thus, it is necessary to maintain specific rates of descent of temperature to avoid functional and structural damage to

sperm. In the first stage of refrigeration, the semen is cooled from 37° C to room temperature, which is not a critical decrease as long as semen is properly extended. However, when the sample is at a temperature below 20.7° C, the lipids from sperm membrane change from the liquid-crystalline to the gel state, resulting in rigid and parallel fatty acids chains [6]. Because of this, once semen reaches 20°C, cooling rates should be increased to 5° C to minimize damage that may affect fertility [7]. The damage resulting from this process are referred as "cold shock" [7] (Figure 1) and are characterized by the abnormal pattern of movement, decreasing of sperm motility, membrane damage and loss of intracellular components [8].

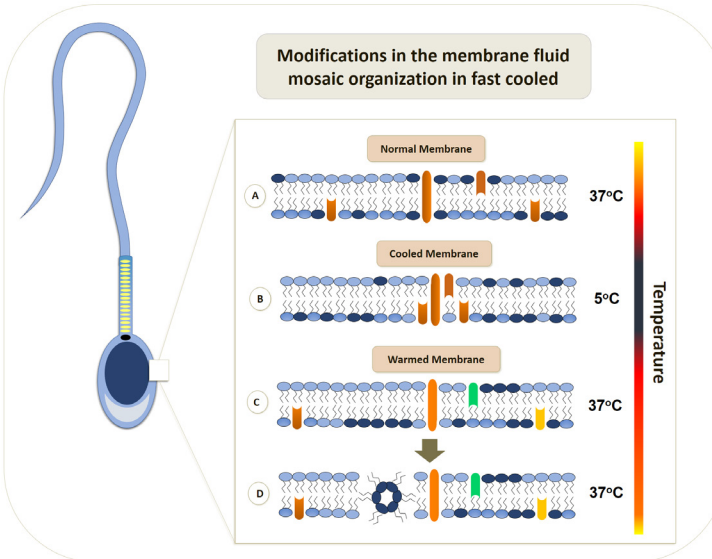


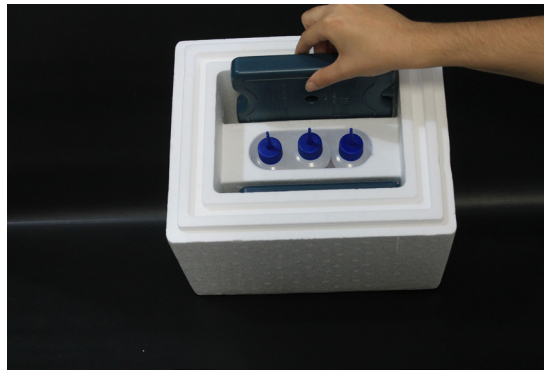
Figure 1: The figure illustrates the changes in the membrane fluid mosaic organization in a fast cooled "cold shock". A) Normal plasma membrane at 37° C; B) The lipid membrane changes from liquid-crystalline state (37° C) to a gel state (5° C); C) The rewarmed plasma membrane with changes in fluid mosaic organization and function of proteins and D) Plasma membrane damage.

Any modifications in the membrane fluid mosaic organization, such as asymmetries and interaction with proteins, may cause changes in its function [9]. Moreover, acrosomal rupture [10] and

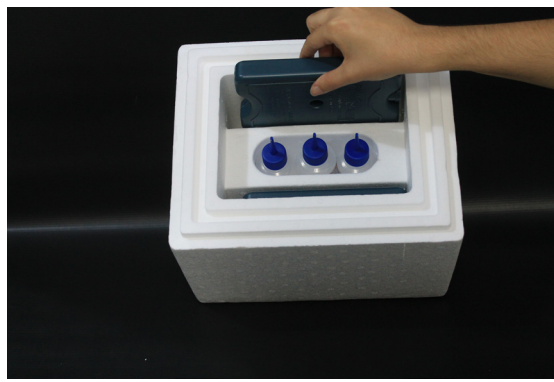
early capacitation may act negatively on the conception rates when using cooled semen. Therefore, it is important to obey specific cooling curves to avoid irreversible damage to the sperm cell [11].

Usually, the cooling of semen prior to insemination should not exceed 48 hours since the sperm viability declines significantly after this period [12], however, the use of new substances in the extender has enabled to store the semen for a longer time [13]. The decrease in semen temperature can be performed in an active or passive cooling system (Figure 2). More commonly, equine semen is transported in passive systems due to its lower cost than that of the active systems however, the proper functioning of the passive systems are dependent on some factors, such as the ambient temperature and initial temperature of the sample [14]. Specifically, these systems shall maintain a specific cooling curve which may be hastened in the range of 37 to 19° C (-0.7° C / min) and then slowed in the range of 19 to 5° C (-0.05 ° C / min).

In addition to the rate of cooling, the passive systems utilize extenders containing lipids, cholesterol, and lipoproteins that may minimize cell damage [15,16]. Several extenders have been developed to maximize the longevity of cooled equine semen, with most considered adequate in maintaining semen at temperatures of 4 to 8°C, and are based on skim milk, glucose and antibiotic [11,12]. The extender addition provides protection to sperm and prolongs its survival during refrigeration and transport [17]. However, for this effect to occur properly a dilution rate of 25-50 x 10^6 sperm per ml is necessary [18]. The maintenance of 5 to 20% seminal plasma in the diluent, which represents dilution rates of 1:4 to 1:19 (Figure 3), has shown to be beneficial in maintaining sperm kinetics for 72 hours of refrigeration [4].



2(A)



2(B)

Figure 2: Passive cooling systems (BotuFlex™): A) Placement of one ice pack for semen shipment at 15°C; B) Placement of two ice packs for semen shipment at 5°C.

Once semen is extended, the recommended insemination dose to obtain satisfactory fertility rates in horses is in the range of 250 to 500x10^6 progressively motile sperm. Because of this, a greater number of sperm is used in the sample for the cooling process to ensure that at least 500 x 10^6 viable sperm remain for insemination [18]. When semen is stored for more than 36 hours, the use of 1 to 1.5 x 10^9 sperm

is recommended because a reduction of 50% of motility is expected [19]. Lower insemination doses can be used in stallions that present good resistance to refrigeration using deep horn insemination [20]. In addition, some stallions present no resistance to sperm cooling [21]. This feature not only depends on the semen quality but also on the composition of the seminal plasma and of the plasma membrane [8].

3 (A)

3(B)

Figure 3: A) Skim-milk based extender addition (Botusemen™); to sêmen; B) Packaging the seminal sample in a plastic container and in a syringe for shipment of refrigerated semen.

The table 1 shows the factors that can influence in the fertility of cooled semen in equines as storage temperature, storage time, extender medium and insemination dose.

Table 1: Fertility of cooled equine semen.

Authors	Storage temperature	Storage time	Semen extender and processing	Fertility rate	Insemination dose
Heiskanen et al, [22]	5-7°C	24 hours	KN	82% (9/11)	-
Heiskanen et al, [22]	5-7°C	24 hours	KNTH	70% (7/10)	-
Heiskanen et al, [23]	5-7°C	70 hours	KN	76% (13/17)	2,0 x 10^9 total
Heiskanen et al, [23]	5-7°C	80 hours	KN	57% (13/23)	2,0 x 10^9 total
Rigby et al., [24]	4 °C	48 hours	CST / 20% SP	72,% (13/18)	250 x 10^6 total
Rigby et al., [24]	4 °C	48 hours	KMT / 0% SP	76% (13/17)	250 x 10^6 total
Nunes et al., [25]	2 °C	24 hours	KN	69% (18/26)	650 x 10^6 total
Nunes et al., [25]	2 °C	48 hours	KN	69% (09/13)	650 x 10^6 total
Hartwig et al. [16]	5 °C	24 hours	BTS	44% (11/25)	1,0 x 10^9 motile
Hartwig et al. [16]	5 °C	24 hours	BTS + Cholesterol	76% (19/25)	1,0 x 10^9 motile
Morrell et al. [26]	-	12-24 hours	INRA	45 (37/82)	0,6 - 1 x 10^9motile
Morrell et al. [26]	-	12-24 hours	INRA + SLC	69% (54/79)	0,4 - 1 x 10^9motile
Neuhauser & Johanes, [27]	5 °C	24 hours	INRA / 0% SP	89% (8/9)	500 x 10^6 total
Neuhauser & Johanes, [27]	5 °C	24 hours	INRA / 5% PS	67% (6/9)	500 x 10^6 total
Neuhauser & Johanes, [27]	5 °C	24 hours	INRA / 20% PS	33% (3/9)	500 x 10^6 total
Neuhauser & Johanes, [27]	5 °C	24 hours	INRA / 80% PS	67% (6/9)	500 x 10^6 total

KN = skim milk-glucose extender, based on the formula published by Kenney et al. (1975); KNTH = Kenney's extender supplemented with 10 mM-theophylline and 10 mM-Hepes, pH 7.2; CST = Commercial skim milk-glucose extender (EZ Mixin-CSTJ, CST, Animal Reproduction Systems, Chino, CA, USA); SP = Seminal plasma; KTM = semen extender supplemented with modified Tyrode's medium; BTS= skim-milk based extender (Botu-Semen™, Botupharma, Botucatu, Brazil); INRA = Semen extender (INRA96; IMV technologies, l'Aigle, France); SLC = Single layer centrifugation.

Seminal plasma (SP) plays an important role for the sperm to reach the uterus during natural coverage. However, many studies have questioned the need of SP for the fertilization process, since sperm recovered from the caudal epididymis present high conception rates [28,29]. Despite the reported benefit in natural coverage, there are reports that SP is deleterious to semen preservation and fertility of fertile stallions [30,31] and subfertile stallions [32]. It should be noted that the influence of SP on fertility rates has great divergence among the surveys. Some authors report that the total withdrawal of SP from ejaculate has a deleterious effect on sperm quality and maintaining 5 to 20% of this fluid in the cooled semen is necessary to increase the fertility and sperm viability [4,33]. In contrast, no difference was observed in the sperm kinetics of cooled semen obtained from good cooler stallions regardless of the addition of SP [34]. However, others found a deleterious effect of seminal plasma on sperm quality and viability in poor cooler stallions, and once the seminal plasma is removed, sperm kinetics and longevity of preserved semen is improved [21,34].

Love et al. [35] observed that in bad cooler stallions there is a significant increase in sperm DNA fragmentation rates after 20 hours of refrigeration, whereas, in good cooler stallions this finding occurs only after 46 hours of refrigeration.

There are two main ways to remove seminal plasma from the ejaculate of stallions: by centrifugation [36] and by filtration [34]. Centrifugation is the most used technique, however, it has the disadvantage of damaging the sperm by mechanical shock, resulting in decreased viability [36] .Thus, the centrifugal force must be adjusted carefully for a given time. Dell'aqua et al. [37] found the association of 600 x g for 10 min centrifugation resulted in 87% recovery of sperm with good quality. In this study, the authors concluded that high centrifugal forces can be deleterious to sperm integrity while low centrifugal forces provided low sperm recovery. The other method of SP

removal is by filtration using a specific filter (Sperm Filter®, Botupharma, Botucatu - Brazil) that draws the seminal plasma through the filter, leaving the spermatozoa concentrated on the membrane surface [38]. This technique causes less damage to the sensitive semen compared to centrifugation [34]. Another alternative to minimize the deleterious effect of SP in the cooled semen sample is the addition of extenders.

Frozen Semen

Cryopreservation of sperm consists of storing the semen at a temperature of -196° C in order to stagnate sperm metabolism, thus increasing cell longevity [39]. In mammals, this biotechnology has been studied for decades, either for the preservation of endangered species or for the diffusion of genetic material from breeders considered superior. Cryopreservation technology was initiated in 1948 by the discovery of glycerol as a cryoprotective cell substance by Cambridge scientists in England [40] and initially the research focused on cryopreservation of bovine spermatozoa. While good results were noted in the bovine, the same success was not obtained for the equine species, and it was found that equine spermatozoa are more sensitive to the freezing process [1]. The first pregnancy obtained with cryopreserved equine semen was not obtained until 1957, using epididymal sperm [41].

Semen freezing has many advantages, since it optimizes the use of the stallion, leading to a higher number of offspring with high genetic value. It allows the genetic material preservation for unlimited time, reduces the costs and risks involved in transporting animals, minimizes the spread of diseases, eliminates geographical barriers, and allows the use of stallions that are unable to mate for physical reasons or are deceased [15,42,43]. However, the use of this biotechnology in the equine species has been limited by the common decrease in post-thaw semen fertility of some stallions, in addition to the increase in foal cost and the need of a greater monitoring of the mare estrous cycle [44,45].

There are also individual and breed factors related to equine sperm resistance to freezing, and as the result of this, the biotechnology is quite variable among stallions [1,46,47]. Sometimes differences can be noted among ejaculates from the same horse [15]. Therefore, stallions can be classified as good freezers or bad freezers, according to the characteristics of post-thaw sperm motility [47]. Stallions that present spermatozoa with post-thaw progressive motile less than or equal to 35% in three or more sample evaluations of a total of ten ejaculates are considered sensitive to cryopreservation (bad freezers), while stallions above this criteria are designated resistant to cryopreservation (good freezers) [48].

The use of frozen semen in artificial insemination programs results in an average pregnancy rate per estrous cycle of about 40%, however this rate may vary from 0% to more than 70% [1,2,49]. Thus, several studies have been carried out to investigate the factors that are related to viability of semen after cryopreservation in horses [33]. Besides the individual differences, several other factors may affect AI success with the use of equine frozen semen, such as the processing, the semen extender, storage system, thawing protocol, and the insemination time, dose, and site in the female genital tract [1].

Because of this, several modifications have been made in the frozen semen processing and insemination protocol in order to improve the results for this species. This includes the use of different extenders [50,51], the addition of different cryoprotectants, the incorporation of lipids [52], the addition of new antioxidants [53,54], of antibiotics [55,56] and of motility stimulants to the semen extender [57,58], as well as the use of different freezing protocols [59,60] of different insemination dose [47,61,62] and methods of insemination [2,63,64].

The cryopreservation and use of frozen semen involves a series of important steps. First, semen collection is performed on the stallion, and the gel fraction is removed via filtration. Semen is immediately evaluated and the initial semen extension is performed with a skim-milk based extender. Following extension, seminal plasma is

removed, and the sperm pellet is resuspended in an extender specific to freezing. Seminal plasma removal is an important step in cryopreservation process to avoid damage to the quality and longevity of semen stored for long periods [65] and can be performed by centrifugation or filtration [66] (Figure 4).

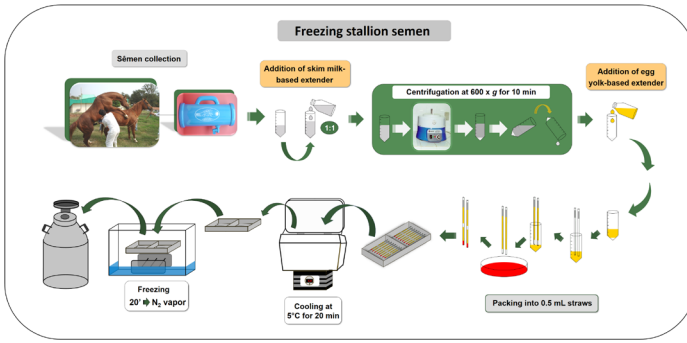


Figure 4: Resume of a manual protocol of freezing semen.

There are several commercial equine freezing extenders and, although they have particularities and specific protocols for freezing, all of them basically aim to balance the pH of the extended semen (buffer effect) by neutralizing toxic products from sperm metabolism, to protect the sperm against thermal shock, to maintain electrolyte and osmotic balance, to inhibit bacterial growth, and to provide energy to the sperm. So, they are composed of tampons, of non-penetrating cryoprotectants (lipoproteins and di or trisaccharides), of energy source (monosaccharides), of intracellular cryoprotectants (such as glycerol and amides) and of other additives such as enzymes, electrolytes, detergents and antibiotics [15]. Penetrating and non-penetrating cryoprotectants are essential for the success of cryopreservation as they prevent ice crystals formation, both within and external to the cell. In addition, cryoprotectants promote an osmotic effect, inducing sperm dehydration, which is essential to their survival.

Although glycerol is the most widely used cryoprotectant in mammalian sperm cryopreservation, other cryoprotectants have been used for equine semen freezing, such as methylformamide, dimethylformamide and dimethylacetamide,, all of which have showed adequate results [44,67]. These amides have a smaller molecular structure than glycerol, penetrating the sperm membrane faster, reducing the need for a long equilibrium time, and thus reducing the cell time exposure to cryoprotectant toxic effect [68]. It should be noted, that although essential to the freezing process, cryoprotectants may cause toxic effects and reduce fertility rates when present at high concentrations [69]. The final step of cryopreservation occurs when straws of semen are stored in cryogenic tanks which contain liquid nitrogen. Following storage, semen is thawed and evaluated to assess longevity and post-thaw kinetics [70].

The freezing process begins with the cooling of the semen from room temperature (around 20° C) to 5° C. At this temperature range the cellular plasma membrane changes from the liquid-crystalline to the gel state and because of this, becomes more susceptible to damage caused by thermal stress. In addition, this causes the semen to lose motility and fertility potential. However, this effect can be minimized by controlling the cooling rate and by adding cryoprotectants to the semen extender. The ideal cooling rate should be slow enough to allow sufficient cell dehydration and avoid intracellular ice crystals formation, but fast enough to avoid prolonged exposure of sperm to hypersaturated solutions from extracellular environment [7,69]. Cell cytoplasm generally freezes at temperatures below -1° C. However, in the range of -10° C to -15° C, even with the frozen extracellular environment, the intracellular environment is still supercooled [6]. In the range of -15 to -60 the complete freezing of the spermatozoa occurs, the metabolic activity ceases and the cell remains inactive. At this stage, the cell must have dehydrated sufficiently to avoid the formation of large ice crystals, but not excessively so, thus avoiding the toxic effect of cryoprotectants and cellular damage [71,72]. After this step, the straws are immersed in liquid nitrogen and are kept at -196° C for an unlimited period of time [15].

The final result of cryopreservation is observed after thawing, which should maintain a curve consistent with that used for freezing. For example, if the freezing curve was slow than recommended, the thawing curve should also be slow so that the extracellular ice crystals thaw, causing dilution of the solutes and promoting slow cell rehydration [6]. Several thawing curves were proposed, including the 37º C curve for at least 30 seconds [73], 46º C for 20 seconds [37] or 75º C for 7 seconds [74]. However, the most commonly used protocol is the first mentioned, as 37º C does not lead to the denaturation of sperm components if the exposure time is exceeded.

The optimal sperm number per straw varies among laboratories and the recommendations of the manufacturer of semen extender, varying in the literature from 100 x 10^6 / ml [60] to 400 x 10^6 / ml [61] using 0.5 ml straws. The inseminating dose may vary according to the post-thawing sperm quality. It is recommended that an inseminating dose using thawed semen should contain at least 150 x 10^6 sperm when post-thaw motility is 35% [47]. However, when the semen quality is inferior the inseminating dose should be increased and a total of 800 x 10^6 sperm may be required [60]. In addition, the ideal AI time using frozen-thawed semen is thought to be between 12 hours before and 4-8 hours after ovulation, as the longevity of cryopreserved sperm is short and the oocyte remains viable within the oviduct for up to 8 hours [75,76]. AI with cryopreserved semen into the mare genital tract can be performed at the uterine body or at the uterotubal junction (ipsilateral to ovulation) using a flexible pipette or hysteroscope. Insemination in the uterine body requires a higher sperm dose, induces greater uterine inflammatory reaction and leads to a delay in the sperm uterine transport to the oviduct [77]. In contrast, Rigby et al. [64] demonstrated that depositing semen in the tip of the uterine horn resulted in a greater proportion of sperm being recovered from the ipsilateral oviduct (77% of recovered sperm), than when sperm were deposited in the uterine body (54% of recovered sperm). Higher sperm colonization in the oviducts reflects higher fertility rates compared to the prior technique [64].

Each step of the cryopreservation process is a critical point to obtain good results with this biotechnology. Success in the use of this technology is effected by the quality of the fresh semen, the interactions between spermatozoa and the semen extender, the cryoprotectants, and the freeze-thaw curve used in order to minimize damage from thermal shock, excessive dehydration, toxic stress, and formation and dissolution of ice crystals [69,78]. Cryopreservation has been reported to cause changes in cell membranes, effect denominated as cold shock [79]. This effect occurs when the sperm are submitted to non-physiological temperatures leading to changes in the lipid two-dimensional organization from plasma membrane, including lipid loss, fluidity and permeability alteration [80]. These damages impair the sperm function [81], reflecting on decreased motility, viability and fertility potential [47,82].

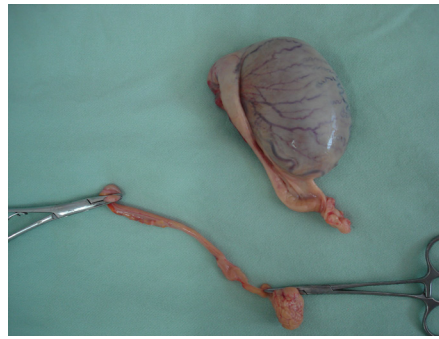
In addition, the sperm resistance to cryoinjury depends on numerous factors, such as the composition of sperm membranes [81,83], the cholesterol/phospholipid ratio of the cell at low temperatures [79,84,85], and the proportion of phospholipids. Therefore, the predominance of phosphatidylcholine increases resistance while the predominance of phosphatidylethanolamine decreases resistance to cryopreservation [86,87]. It should be noted that these features of sperm membrane composition are genetically transmitted [88].

Despite many advantages, the equine semen cryopreservation faces lower fertility rates than those found with fresh and cooled semen. Cryopreservation is a process that depends on several complex biochemical steps and events, most of them not yet completely elucidated, which will influence cell viability at the end. Therefore, several studies have been carried out in order to clarify the mechanisms involved and to test new substances and protocols to improve the results with this technique, aiming to increase its use in the future.

Preservation of Epididymal Sperm

Sudden death, catastrophic injury, castration, obstructive processes or any other event that precludes semen collection or mating may prematurely terminate a stallions reproductive life. In these cases, a final sperm collection may be necessary to preserve the genetic value of an animal. Because of this, studies on the preservation of epididymal sperm for future use have been intensified to prevent genetic losses. These investigations have described techniques for epididymal sperm collection [89,90], laboratory procedures such as the use of different extenders for flushing and cryopreservation [28,91], the addition of seminal plasma [43,92-94], insemination [29,91]and assisted intracytoplasmic sperm injection [95].

There are several methods of equine epididymal sperm recovery. These can be divided into sperm recovering "*in vivo*" and "*in vitro*", however the most used techniques are "*in vitro*", and in particular the flotation and retrograde flow methods. The flotation method consists of performing longitudinally incisions in the cauda epididymis, exposing the lumen of the tubule and allowing the output of sperm. Afterwards, extender is added to help in the recovery and dilution of cells [96]. In contrast, the retrograde flow technique consists of isolation of the epididymis from the testis, the removal of the tunica albuginea and a careful dissection of the connective tissue around epididymis (Figure 5). In both, the single tube is entirely flushed by the extender starting from the vas deferens after uncoiling the epididymal duct, allowing for sperm output up to the epididymis tail-body transition [97] (Figure 6). The average sperm recovery using this method ranges from 12.5 to 20 billion sperm per epididymis [29,90].



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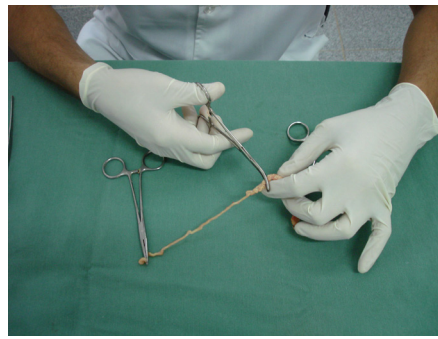

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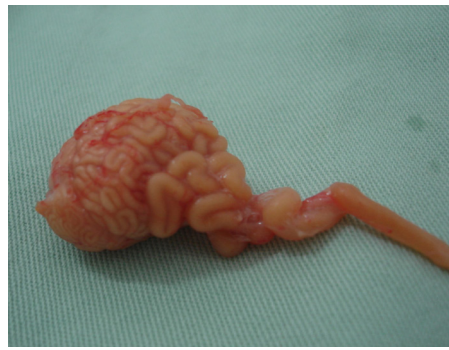

C

Figure 5: Steps that precede epididymal sperm recovery by the retrogade flow technique: isolation of the epididymis from the testis (a); removal of túnica albuginea and connective tissue around epididymis (b); convolutions of cauda epididymis (c).

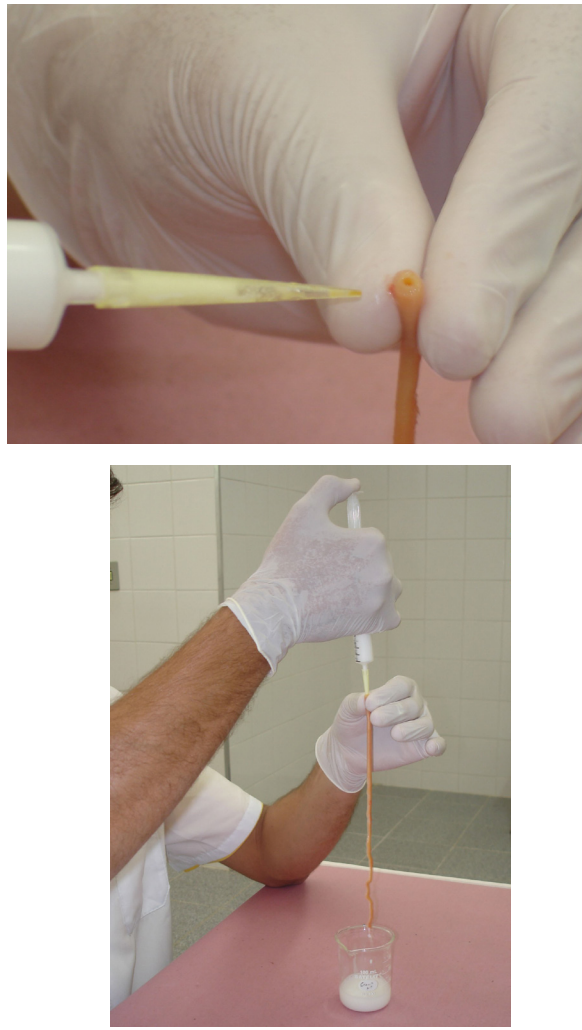

Figure 6: The epididymal duct is uncoiled and entirely flushed by the semen extender, allowing the sperm output.

After removal of the epididymis, sperm remain viable until affected by tissue decomposition [98,99]. Sperm viability is maintained for longer periods when the epididymis is stored at 5°C [29,98-100]. If the epididymis is maintained at room temperature, there is a progressive decline in sperm quality, surviving for up to 24 hours after orchiectomy. This decrease may be explained by both the aging and metabolic depletion of sperm but also the process of postmortem tissue degeneration.

Nonetheless, storage at 5° C for up to 96 h proved to be efficient in maintaining sperm quality [99,101] and did not affect progressive motility and membrane integrity after thawing [101]. In addition, the maintenance of the testis-epididymis complex at 5° C for 24 h before sperm recovery showed the same conception rates when compared to natural ejaculate (61.5%) and epididymis sperm recovered immediately after orchiectomy (92.3 %) [29].

In horses, epididymal sperm have been shown to be resistant to cryopreservation enabling the use of breeders that had already been retired or even have died, by AI techniques such as conventional artificial inseminations [28,29,32,91], by hysteroscopy [92] or using ICSI [95]. Fertility rates using epididymal semen have remained low for many years and this technology has only recently achieved better results by changing freezing protocols, changing the timing of insemination, and adding substances to epididymal sperm (Table 2) [29,91,94]. Analyzing fertility, Morris et al. [92] evaluated the effect of exposure of epididymal sperm to a motile stimulant (Sperm-TALP). Inseminations were performed by hysteroscopy, using single thawed doses of 0.5 mL containing $5x10^6$ motile sperm. In this study, no pregnancies were observed in the groups without motile stimulant whereas 29% of mares became pregnant when epididymal sperm were obtained with Sperm-TALP was added.

Heise et al. [94] compared the effect of the seminal plasma (SP) addition on the conception rate of sperm recovered from the cauda epididymis. The fertility rates obtained using seminal plasma addition

were 55.6% for fresh epididymal sperm and 38.9% for frozen epididymal sperm. In the absence of seminal plasma, these values were 22.2% and 6.7%, respectively. This study demonstrated the greater fertilization capacity of equine sperm obtained from the cauda epididymis after addition of seminal plasma.

In subfertile stallions, the contact of sperm with seminal plasma during ejaculation can have a deleterious effect on sperm quality. In these stallions, the total motility, progressive motility and plasma membrane integrity of sperm obtained from the cauda epididymis without seminal plasma contact, were superior to sperm from ejaculate before and after freezing [32].

Epididymal sperm can also be used in biotechnologies such as intracytoplasmic sperm injection (ICSI), presenting no differences in the proportion of embryonic cleavage when compared to cryopreserved sperm from ejaculate of stallions [95].

Table 2: Fertility of sperm recovered from the epididymis.

	Authors	Epididymal sperm categories	Dose insemination	Pregnancy rates	Method of insemination
1	**Barker & Gandier [41]**	Frozen/Thawed	No described	12,5%	Uterine body
2	**Morris et al, [92]**	Fresh	$200x10^6$	45%	Hysteroscpic
		Frozen/Thawed	$200x10^6$	8%	Hysteroscpic
		Frozen/Thawed	$200x10^6$	18%	Conventional
		Frozen/Thawed	5-10 x 10^6	0%	Hysteroscpic
		Frozen/Thawed + ST	5-10 x 10^6	28%	Hysteroscpic
3	**Papa et al, [28]**	Frozen/Thawed	$800x10^6$	66,6%	Uterine horn
4	**Heise, [94]**	Fresh + SP	$200x10^6$	75%	Uterine body
		Fresh - SP	$200x10^6$	22,2%	Uterine body
		Frozen/Thawed + PS	$200x10^6$	27,8%	Uterine body
		Frozen/Thawed - PS	$200x10^6$	6,7%	Uterine body
5	**Monteiro et al, [29]**	Frozen/Thawed	$800x10^6$	91,3%	Uterine horn
		Cooled+Frozen/Thawed	$800x10^6$	61,5%	Uterine horn
6	**Monteiro et al, [32]**	Frozen/Thawed/Subfetile	$800x10^6$	25,0 %	Uterine horn
7	**Monteiro, [102]**	Frozen/Thawed/Subfetile	$800x10^6$	48,4 %	Uterine horn
8	**Guasti et al, [91]**	Frozen/Thawed	$800x10^6$	68,7%	Uterine horn
		Frozen/Thawed	$100x10^6$	31,5%	Uterine horn
		PTX + Frozen/Thawed	$100x10^6$	50,0%	Uterine horn

Cooled = Prior to epididymal sperm recovery, the testicular-epididymis complex was stored for 24 h at 5° C; SP = Cauda epididymis was flushed with seminal plasma; ST = Samples were resuspended in sperm talp before freezing; PTX = Tail epididymis was flushed using skim-milk-based extender containing 7.18 nm of pentoxifylline.

New Biotechnologies of Semen

The new biotechnologies of semen aim not only to improve the quality of cooled and frozen semen, but also to increase the semen quality and fertility of stallions considered subfertile. Stallions represent 50% of the breeding equation, and the horse industry is replete with stallions whose level of fertility is undesirable. This issue may be due to the criterion for equine breeder selection that generally considers only the pedigree, performance record, and breed conformation, leaving aside the reproductive efficiency [103]. In this sense, seminal plasma removal techniques minimizing the impact of centrifugation, and sperm selection were developed to allow the use of ejaculates sensitive to the cryopreservation or with low quality [33].

Seminal Plasma Removal Techniques less Damaging to Sperm

Seminal plasma is usually removed from semen during cryopreservation process, as several studies have demonstrated the deleterious effect of this fluid on motility, DNA integrity and plasma membrane integrity during storage [104-107]. Therefore, its removal via centrifugation was introduced as an efficient method to concentrate the sperm in a pellet and separate the seminal plasma [108]. However, the velocity and duration of centrifugation may adversely affect the total number of sperm, promoting mechanical damage to the plasma membrane and increasing some morphological defects that result in decreased sperm viability [36,109,110]. Therefore, the development of high viscosity colloidal solutions, inert and isotonic, which function as a cushion at the bottom of the tube during centrifugation (Equicellsire®, IMV, L'Aigle, France, Cushion Fluid®, Minitube, Landshut, Germany, Red Cushion®, Botupharma, Botucatu, Brazil) have been developed to improve the outcome of cryopreserved semen in recent years by minimizing the impact and compaction of sperm [33,111]. The cushion technique increases sperm recovery as it enables the use of a higher centrifugation velocity (800-1000 × g) compared to conventional centrifugation without cushion (300-500 × g), without leading to damane of the sperm structure [112,113].

An alternative to centrifugation is the use of a hydrophilic synthetic membrane filter (Sperm Filter, Botucatu, Botucatu, Brazil) that uses surface tension to draw the smaller molecules of seminal plasma and other structures lower then 2 μm through the filter (Figure 7), leaving the spermatozoa concentrated above [34,38,66]. The advantages of this technique include a higher sperm recovery rate, decreased cost, readily useable with minimal training and less detriment to sperm viability compared to traditional centrifugation [66]. Also, Ramires-Neto et al. [114] demonstrated that the removal of seminal plasma by filtration reduced the bacterial load on the semen after cooling. However, centrifugation using a cushion was a superior method to remove seminal plasma compared to filtration for total and progressive motility of cooled sperm [115].

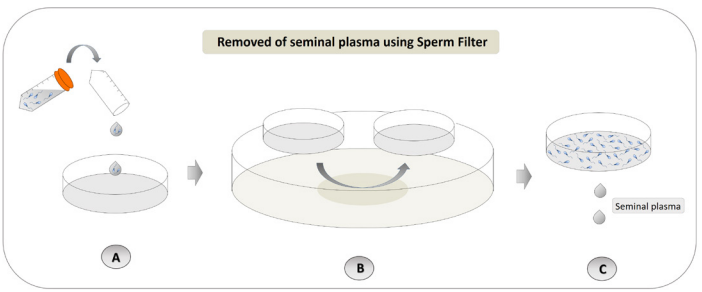

Figure 7: Illustration of seminal plasma removal technique by filtration using a hydrophilic membrane.

Spermatic Selection Techniques

Some forms of subfertility may have a genetic basis, but it is often associated with aging or acquired diseases in stallions. Among the acquired diseases that affect sperm quality, the most frequent is testicular degeneration that generally occurs after environmental stress, fever and genital trauma, or by the use of long-term medications such as anabolic steroids [103]. Besides subfertility, some stallions exhibit

a decrease in sperm quality after cooling or cryopreservation due to greater sperm sensitivity, as previously mentioned [15].

Sperm selection techniques are used to enrich a semen sample with a higher percentage of viable sperm before mare insemination. This may refer to poor quality fresh ejaculates or damaged spermatozoa after cooling or freezing [103,116]. Several techniques of sperm selection have been tested for equine semen, such as the motile sperm migration technique (swim-up and swim-down techniques), the sperm adherence technique and the density gradient centrifugation technique [33]. However, the most commonly used technique in improving the quality of equine semen is the single layer sperm selection (Figure 8). There are currently two commercial silica-based products (EquiPure®, Nidacon, Gothenburg, Sweden or Androcoll-E®, Minitube, Landshut, Germany) that perform similarly. In both, the selection of sperm is based on progressive motility, plasma membrane integrity and morphological defects [117,118], reflecting higher fertility rates [119]. Similar to the Sperm Filter, these gradients were efficient in reducing bacterial [120] and viral load [121] after semen processing.

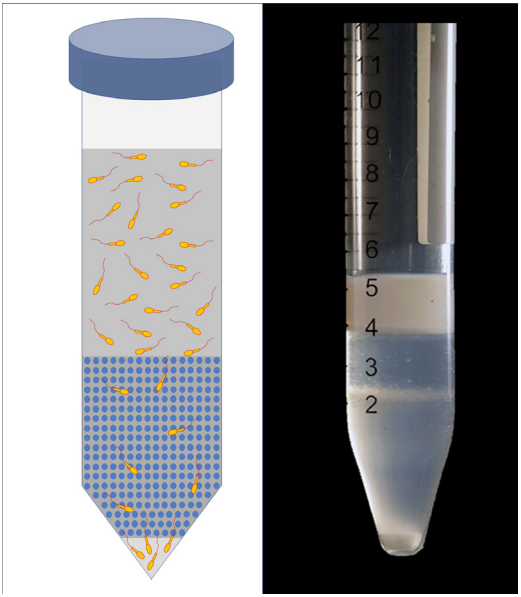

Figure 8: Illustration of the sperm selection in single layer technique.

Summary

Over the years several biotechniques have emerged and gradually were incorporated into veterinary practice. Particularly, semen biotechniques have arisen in order to increase semen longevity and the reproductive efficiency of stallions with superior genetic merit. These technologies are even more important for horses, because the breeding selection rarely includes fertility parameters, resulting in a large number of subfertile stallions. Thus, the increased fertility of these animals and the improvement of the quality of sensitive semen to storage result in a favorable economic impact on the horse industry.

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Chapter 10

Reproductive Biotechnologies of Buffaloes

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Abstract

Buffaloes are easily adaptable to remote environments and to forage that would be of low quality for cattle. Initially, buffalo farming was implemented to occupy areas not well explored. As a consequence, buffaloes became an important economical resource for small farmers. Additionally, buffaloes have a high feed conversion efficiency and are able to better utilize lower quality food products for meat and milk production. Consequently, there is a growing interest in buffalo production in the current world economic scenario. This leads to investments in biotechnology programs similar to those used with bovines, to improve genetic and reproductive parameters in buffalo herds. However, most of these programs are not economically viable yet, as the results obtained with biotechnological programs for buffaloes are inferior to those obtained with bovines. Accordingly, there is an increasing demand for studies about buffaloes, with the objectives of increasing the viability and results obtained with the use of reproductive biotechnologies with these animals. Therefore, the objective of this chapter is to review the most important reproductive biotechnologies for male buffaloes.

Specific Characteristics of Buffaloes

Before we discuss how to implement reproductive biotechnologies to improve buffalo reproductive efficiency, it is essential to understand basic reproductive characteristics that differentiate buffaloes from bovines.

The domestic buffaloes (*Bubalus bubalis*) belong to the family and subfamily Bovidae, and to the genus bubalis. They are classified in two subspecies: *Bubalus bubalis carabenesis* and *Bubalus bubalis bubalis*, respectively known as swamp buffalo (48 chromosomes) and water buffalo (50 chromosomes). Swamp buffaloes are mainly used for meat production. These animals are also commonly used for traction power in rice plantations at swampy areas. On the other hand, water buffaloes have a double aptitude, being used for meat and milk production [1].

Puberty in males is characterized by an ejaculate containing approximately 50 million total sperm per mL with progressive motility higher or equal to 10% [2]. On the other hand, sexual maturity consists of the moment when the animal completely develops secondary sexual characteristics, maximum libido and seminal quality compatible with good fertility rates [3].

Male buffaloes reach puberty approximately between 12 and 14 months of age (Figure 1) and sexual maturity between 24 to 30 months [4,5]. Feeding systems, management, breed and even individual differences account for the large variation in age at which the animals reach puberty and sexual maturity. In general, buffaloes are sexually mature later than cattle [6].

Figure 1: Herd of Murrah bulls (*Bubalus bubalis*) at the beginning of puberty.

Buffalo heifers attain puberty when their first estrus occurs, associated with ovulation and the formation of a corpus luteum [7]. Females are approximately 26 months of age at puberty [8]. However, the presence of estrus without ovulations might occur earlier [8,9]. The duration of the estrous cycle of female buffaloes varies from 16 to 33 days, occurring more frequently every 21-24 days [7].

Buffaloes have reproductive seasonality, directly influenced by the photoperiod (Figure 2). Additionally, buffaloes are very sensitive to heat stress, which negatively influences their reproductive activity [10]. In regions that are distant from the equator there is an interruption of cyclicity during the summer, when there is daily light for longer periods of time. Consequently, there are more estrous cycles during the colder seasons of the year [11]. However, in regions near the equator females can exhibit a seasonal polyestrous behavior [7]. In males, although the spermatic production happens during the entire year, there is higher seminal quality during the fall and winter [12] and there is a decrease in the libido during the spring and summer [13].

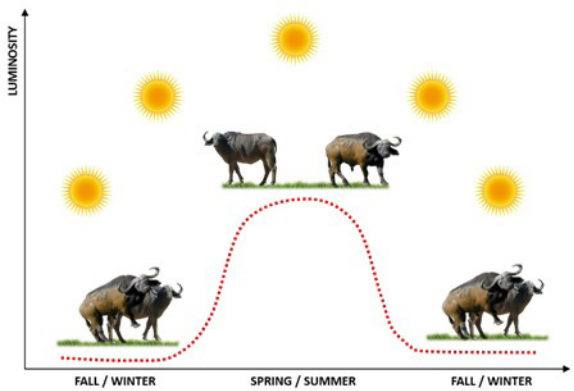

Figure 2: In regions with well-defined seasons of the year, during the spring and summer, when there is a longer daily period of light, there is an interruption of the cyclicity of the female and a decrease in libido and seminal quality in the male. In the fall and winter, when the period of daily light is shorter, there is a return of the female cyclicity, increase in libido and seminal quality in the male. This is the period of reproductive activity of the species.

Pasture Breeding and Controlled Pasture Breeding

Buffalo production is mainly based in the extensive management system, with pasture breeding being the most employed system in this type of production. Thus, it is necessary to evaluate the males before the breeding season, so that they are classified as apt or inapt for reproduction through a complete andrological examination [14].

It is well known that buffaloes have low libido when compared to bovines [15] because of lower concentrations of circulating reproductive hormones [16]. With a pasture breeding system it is important to take into consideration the proportion of male/female and age of the bulls, so as to use the full breeding capacity of the bull with good fertility rates [3]. For young bulls, 2 years of age, the ratio should be of 1 bull for every 15 to 25 heifers; for animals with 3 years of age the ratio can be of 1 bull for 25 to 35 heifers; breeders of 4 years can use a ratio of 1 male to 35 to 45 heifers and for males older than 5 years 1 bull for 45 to 60 heifers. These numbers can be increased when a controlled pasture breeding system is used in which the bull can mate with approximately 120 heifers during one breeding season. In this system, the bull is kept separate from the females until estrus is detected, then they are inserted in the herd [17].

Buffalo bulls manifest their sexual behavior during the mating process as they approach the female in estrus. The common and desirable traits of a breeding bull include the Flehmen reflex, projection of the penis a few centimeters associated with tactile penile stimulation and olfactory contact with the female and the detection of pheromones through the vomeronasal organ. Additionally, it is common to observe the male resting his chin on the female, to verify that the female will accept the mount [18-20].

Reproductive Biotechnologies

Buffalo farming has an important role for food production in many developing countries, especially in South Asia and Mediterra-

nean regions. The buffalo population is estimated to be 199 million and there is a growing demand to increase this population and its productivity [21]. To accompany this growth and improve herd quality, biotechnologies used for bovines have also being implemented to bubalinoculture [22]. However, the efficiency of most of these biotechnologies is still low in buffaloes [23]. Although the reproductive biology of buffalos resembles that of cattle, there are important differences and particularities that need to be considered in order to apply biotechnologies more efficiently, thus improving reproductive productivity in buffaloes [24].

Semen Biotechnologies

Semen Collection

Semen from buffaloes can be collected using electroejaculation, artificial vagina (Figure 3) or transrectal massage of the ampullary glands [25]. Among the available methods, collection using an artificial vagina (Figure 4), on a phantom or on a female with sexual receptivity, is the most advantageous, since it mimics the conditions of natural mating. This method results in an ejaculate with a higher volume, concentration and percentage of motile spermatozoa, in addition to a smaller amount of seminal plasma. Also, male buffaloes are easily conditioned to semen collection with an artificial vagina, as long as the device is maintained at a temperature between 39ºC and 42°C [12,26-28].

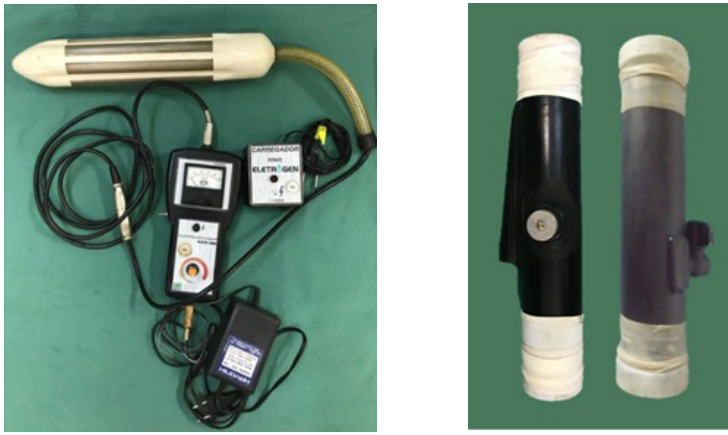

Figure 3: Electroejaculator used to collect semen by electroejaculation method; and artificial vaginas commonly used to collect semen on a mannequin or on a female in sexual receptivity.

Figure 4: Semen collection using artificial vagina with a female in sexual receptivity.

Most buffalo bulls do not respond well to the electroejaculation method because they are easily stressed by this type of manipulation and many do not ejaculate under the electric stimulus. Additionally, the method results in a semen with inferior quality when compared to the artificial vagina collection, as the ejaculates are usually very di-

luted and can be contaminated with urine [27]. The semen collection with a transrectal massage of the ampullary glands results in a good quality semen (Figure 5), however, it requires a lot of practice and many animals do not respond to the stimuli [14].

Another semen collection technique tested with buffaloes is obtaining sperm from the caudal epididymis, with excellent results *in vitro* [29-31]. However, additional studies are needed to evaluate fertility of buffalo epididymal spermatozoa. This technique can also be used to recover sperm from animals post-mortem. Therefore, it makes it possible to obtain and preserve samples from animals with an important genetic material and also from endangered species [30].

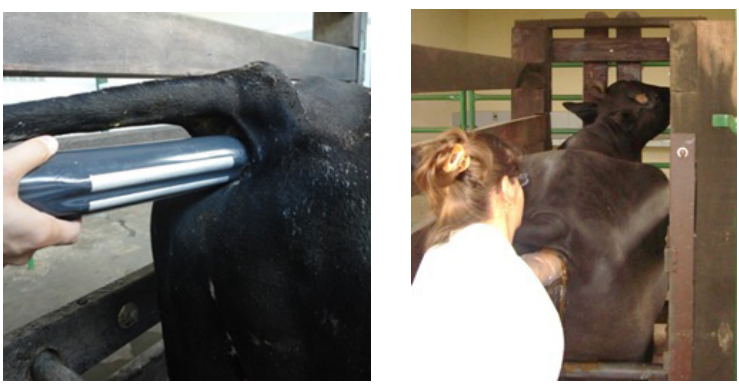

Figure 5: Semen collection by electroejaculation and massage of the ampulla glands, respectively.

After reaching sexual maturity, the buffalo bull can be collected daily and still maintain high fertility rates. When the bulls are collected one or two times per day during short periods, an overnight rest period is sufficient to restore sperm reserves. However, when used in an excessive manner, a longer rest period may be necessary [32].

The normal color of the semen can vary according to their diets, from a white, white-grayish to a yellow citrine color (Hafez and Hafez, 2004). Sperm density varies from an aqueous to a milky, creamy-

milky, or creamy aspect, as the spermatozoa concentration increases [33]. The semen pH varies from 6.4 to 7.5 in this species [34-38].

Seminal volume and spermatic concentration vary depending on breed, age, size, number of mounts, collection method, nutrition, general health and environment [38]. Older animals have a larger seminal volume (4.0 to 8.0 mL) when compared to young bulls (1.0 to 3.0 mL) [14]. The number of mounts also increases the seminal volume, because there is a larger production of seminal plasma by the accessory glands which are stimulated during a longer period of time. However, the number of sperm ejaculated does not increase, reducing the spermatic concentration of the ejaculate [39].

Sperm concentration varies from 600 to 1200 million sperm per mL. The concentration is affected by age, seasonal and nutritional factors of the bull [40].

Mass motility, or swirling movement, varies according to the sperm concentration, percentage of cells with normal forward progressive movement and sperm velocity. Evaluation is done with light microscopy, with a 10x objective, on a non-diluted drop of semen (5-10 μL) in a glass slide at 37°C without a cover slip. The evaluation should be done near the edge of the drop [39]. The scale for the assessment is 1 to 4, where 1 is the absence of movement and 4 is the movement considered as maximum. A good quality ejaculate should not have mass motility lower than 3 and darker and lighter waves are an indicator of a high sperm concentration [33].

Motility is evaluated by considering the percentage of motile cells in the sample (from 0 to 100%) (Barth and Tribulo, 2000) and is routinely assessed by visual estimate with an optical microscope (in 20 to 40x) in the sample at 37°C [12]. Ejaculates considered as having good quality should have at least 70% of motile sperm, while ejaculates with motility lower than 20% should be discarded [42,43].

The spermatic vigour is the speed in which the sperm moves and is classified in a scale of 1 to 5, where 1 is considered as low and 5 as

the maximum vigour. Semen from buffaloes should have a spermatic vigour above 3 [38].

Semen Cryopreservation

The cryopreservation of buffalo semen began in 1972, however, the use of frozen semen in artificial insemination (AI) programs is still one of the obstacles to the success of this biotechnology in buffalo herds due to a reduction in the quality and fertility of the spermatozoa after cryoprocessing. Consequently, several studies have been done to improve the quality of cryopreserved semen in this species, with the development of better dilution media, cryoprotectants and freezing and thawing methods [12,44,45].

The poor freezability of buffalo spermatozoa, when compared to bull sperm, is due to an inherent fragility of the plasma membrane in this species due to the low concentration of phospholipids in their structure which are lost during freeze-thawing [12]. Furthermore, buffalo semen has higher concentrations of calcium, chloride, phosphorus and alkaline phosphatase with lower concentrations of total electrolytes, ascorbic acid and proteins, which contributes to lower sperm quality after cryopreservation [46]. Additionally, buffalo seminal plasma has low concentrations of natural antioxidants, which can be further reduced during dilution and cryopreservation [47].

Semen dilution should be done before freezing and it has several advantages, such as: reduction of bacterial load, supplies energy substrates, protects sperm at unfavorable temperatures, reduction of the toxic effects of byproducts of sperm metabolism and prevention of sperm agglutination [48]. Thus, most semen diluents are composed of liposomes, lipoproteins (from egg yolk or milk), sugars, glycerol and other additives, such as enzymes, electrolytes, detergents and antibiotics [49] at different percentages according to the composition of the diluents. Although they are essential to prevent ice crystal formation, avoiding cryoinjuries, cryoprotectants can cause toxic effects to the sperm, leading to irreversible damage if the physicochemical cell

structure is affected, thus decreasing sperm motility and fertilizing capacity [50].

Egg yolk is one of the most important ingredients in diluents because it works as an extracellular cryoprotectant. It acts by protecting the plasmatic membrane against heat stress and prevents or restores the loss of membrane phospholipids during the cryopreservation process [51]. Among intracellular cryoprotectants, glycerol is the most commonly used for buffalo semen cryopreservation, with optimal levels at 6-7%. Acceptable egg yolk concentrations in diluents are of 20%. The ideal equilibration time should be of 2-6 h, with a slow cooling (0.2-0.4 ° C / min) rate recommended during the pre-freezing process [12].

Another important component in semen extender formulation are buffered mediums, which create a pH adequate for spermatic survival. Among the various substances commonly used, such as the Tris-citric acid, Tris-tes, Tris-hepes, Tris-citrate and Tris-base, the latter two had better results for buffalo semen, with better sperm motility and plasmatic integrity/acrosomal parameters when compared to other buffers [52-54].

Conventional extenders such as the egg yolk citrate or milk that are commonly used for sperm cryopreservation in bovine bulls are considered as suboptimal for buffalo semen. Therefore, several other diluents were formulated in an attempt to improve the results for freezing buffalo semen [44].

Dhami et al. [55] reported good results when using tris-yolk glycerol with caffeine citric acid and raffinose as extender for freezing semen from buffaloes. Zorzetto et al. [56] compared tris-yolk-glycerol, Botu-bov® and ACP-111® (coconut water based) extenders and concluded that the tris-yolk-glycerol and Botu-bov® had the best results when compared to the ACP-111® for sperm kinetic parameters.

Studies have shown that results of using tes-tris-yolk glycerol and mes-tris-yolk-glycerol have been as good as when using other tris

dilutors [52,53]. Confirming these findings, Vale et al. [57] had the same pregnancy rates (mean of 57.1%), using frozen ejaculates with TRIS and TES based media.

The ascorbic acid content in buffalo semen is significantly lower than in bovines. Thus, the addition of antioxidants agents or precursors, such as gluthatione, to the diluent has a beneficial effect on buffalo semen [58,59]. Additionally, other additives such as cysteine hydrochloride, EDTA and membrane stabilizers such as chlorpromazine hydrochloride can also be used to minimize acrosomal damage and sperm capacitation during freezing [60].

The basic principle of semen cryopreservation is to interrupt sperm metabolism, preserving its characteristics for an extended period of time [61]. However, during processing and freezing several factors affect the final sperm quality, directly affecting conception rates in an AI program [62]. Success in semen cryopreservation is closely related to the quality of fresh semen and its interactions with the diluent, cryoprotectants and freeze-thaw cycles used. These processes should be done in a way that minimizes damage from thermal shock, excessive dehydration, toxic stress, and formation and melting of ice crystals [63].

Buffalo semen cryopreservation follows a specific protocol with distinct steps: dilution of the collected semen in a freezing diluent; packed and sealed in mini straws; refrigeration in a refrigerator at 5ºC during 4 hours; the pre-freezing, where the straws are placed horizontally at 1-4 cm above the nitrogen vapour during 10-20 minutes; finally, freezing when semen straws are then submerged into liquid nitrogen at -196 ºC (Figure 6). It is interesting to note, that the freezing process with different steps using a programmable freezer resulted in a better quality semen post-thawing [64].

Figure 6: Straws arranged horizontally before being placed 1-4 cm above the nitrogen vapor for 10-20 minutes; cryogenic containers for frozen semen storage until the moment of insemination; straw containing thawed semen to be used in an artificial insemination.

Usually, the semen is diluted in one or two steps to a final sperm concentration of about 100–150 x 10^6 sperm/ml and packed into 0.25 ml straws [12]. This methodology is used because Haranath et al. [65] reported better conception rates from semen cryopreserved in these mini straws when compared to semen cryopreserved in medium straws (0.5 ml).

The freeze rate must be slow enough to allow the water to leave the cells through osmosis, preventing formation of intracellular ice, but fast enough to avoid an excessive dehydration and toxic damage caused by cryoprotectants [63]. During the refrigeration stage, the plasmatic membrane transitions from liquid-crystalline to gel phase, and the susceptibility of the membrane to submit itself to these transitions is inversely related to the cholesterol and unsaturated fatty acids proportion in the membrane [66].

The best protocol to thaw frozen buffalo semen is by submersing the straw in a warm water bath at 40° C for 30 minutes. Another protocol is to thaw the frozen semen at a higher temperature of 50°C during 15 seconds [55,10].

Effect of seminal plasma removal before cryopreservation was evaluated in buffalos by using centrifugation or hydrophilic membrane filtration [67]. In this study, the authors did not observe improvements in the post-thaw semen quality when compared to the semen that contained seminal plasma. The removal methods used also did not result in a different semen quality.

Seasonality directly affects the quality of the cryopreserved semen and conception rates using cryopreserved semen. Many studies have showed fewer sperm defects [28,68-71], better sperm motility [68,70], integrity of the sperm plasma membrane [28] and DNA integrity [72] of cryopreserved buffalo bull spermatozoa during the fall/winter when compared to semen that was frozen during the summer. Furthermore, the fertility rates from the semen post-thawing is lower during the warmer and humid months when compared to the winter [28].

As cryopreserved buffalo bull spermatozoa has a shorter viable and fertile time in the female reproductive tract, adequate estrus detection and AI at the adequate moment are critical strategies to increase the conception rates in this species when using frozen semen [73].

Cooled Semen

The ingredients used in the formulation of extenders for semen cryopreservation, such as Tris, egg yolk, skim milk and coconut water, are also the ingredients that compose the extenders for storage at 4ºC of buffalo semen [24]. The conception rates using the natural service or inseminating with cooled semen and frozen semen were respectively 60, 35-60 and 25-45% [74,75]. However, buffalo semen is not commonly used chilled, as it can be stored at 58° C for up to 72 hours without significant decrease in motility if the extender presents the same composition as those used for freezing [55].

Sexed Semen

After Painter's [76] discovery that sperm is a carrier of the sex chromosome X or Y, several research have focused on separating the semen into fractions containing only one of these populations, in order to designate the sex of the future individual. Selection of the desired sex of the offspring may be one of the determining factors to increase the genetic progress and profitability of livestock, since it provides high productive efficiency of meat or milk, from characteristics that are limited or influenced by sex [77].

In the dairy industry, this biotechnology is an important tool to increase the supply of replacement heifers by the use of sexed semen containing spermatozoa carrying the X chromosome, accelerating the genetic gain [78]. On the opposite side, the use of sexed semen containing the Y chromosome increases the incidence of male calves, a product of greater interest in the meat industry, due to the higher carcass yield. Also, this semen population is used to increase the population of animals designated for traction [79]. A 15% increase in genetic gain is expected with this biotechnology compared to conventional

semen, in addition to reducing costs with progeny tests and genetic markers [80].

Until the 1990s, the only option for sexual pre-selection was by sexing embryos prior to transfer to the recipient females, however, Presicce et al. [81] pioneered sorted the buffalo semen by flow cytometry. These researchers proved the efficiency of this technique when reporting the calf birth using sexed semen from Mediterranean buffaloes [82].

For many years, several researches have focused on the development of methods for the separation of spermatozoa containing different sex chromosomes, such as by density difference in colloid centrifugation [83], by weight and electric charge difference [84], by differences in protein content [85], by the presence of different antibodies [86] and by differences in the amount of DNA [87]. Flow cytometry is currently the only reliable method for obtaining sexed semen doses, presenting 85-95% accuracy in obtaining offspring with pre-determined sex [88].

In buffaloes, sperm sorting was initially performed by Lu et al. [89,90], which demonstrated the viability of this technique in this species with 94% and 89% accuracy for classification of X and Y spermatozoa, respectively. A few years later, promising pregnancy rates (50%) were achieved by inseminating buffaloes with a dose of sexed semen containing 4 million sperm [91].

Numerous studies have shown that the process of semen sorting using flow cytometry involves a series of steps that can cause irreversible damage to the sperm cell, reducing its viability and longevity mainly in the female reproductive tract [79,92]. Thus, lower conception rates using artificial insemination and in vitro embryo production is observed when compared to the rates obtained with AI using conventional semen. However, the use of buffalo sexed semen for in vitro fertilization resulted in cleavage similar to unsexed semen (approximately 53%), promoting good perspectives in the application of this technology in this species [93,94].

Currently, several studies have been performed to unleash this biotechnology in the buffalo industry. It's use in large scale probably will expand in the future in response to the demand for females and males, if the biological causes of poor embryonic development and compromised conception rates of transferred embryos get to be understood and improved [95].

Artificial Insemination

Artificial insemination (AI) is the most important biotechnology in the propagation of genes in livestock animals and has numerous advantages such as the reduction of risks involved in the transport of animals, reduction of disease transmission, enables a worldwide exchange of genetic material, and mainly accelerates the genetic improvement of the herds. In cattle, it is a biotechnology widely used to accelerate the genetic improvement of offspring, increasing the production of milk and meat, from bulls with tested progeny [96].

The main obstacles to use AI efficiently in buffaloes include the seasonality of this species that interferes with ovulation and estrus manifestation by females and the decline in semen quality and libido in males. There are also factors related to management, such as adequate detection of estrus, which has a short duration and a prolonged ovulatory wave, which makes it difficult to detect the ideal moment to perform AI even during the reproductive season [97]. In addition, the natural narrow cervix of buffalo females makes AI more difficult, requiring experienced inseminators [24].

Failures in semen handling and inadequate post-insemination and postpartum nutrition also contribute to low reproductive rates using AI. Lack of estrus detection is mainly due to the fact that female buffalo show very discrete signs and, most of the time, during the night period [22,96-99].

To achieve success with the use of AI, two or more observations of estrus during the day are required, during the period of the mating season, which demands time and trained observers [98,100]. Fail-

ures in this process will lead to low conception rates and a decrease in reproductive performance, leading to an increased service period and longer interval between deliveries [99,101]. Therefore, other techniques for efficient estrus detection in buffaloes have been developed in order to improve AI efficiency, such as: measure vaginal electrical resistance [102], perform progesterone assay on milk and plasma [103], conduct laparoscopy [104] and ultrasonography [105].

During spring and summer, ,silent estrus and increased ovulation time in buffalo females commonly occurs, mainly caused by caloric stress, resulting in reproductive delays, mainly caused by caloric stress [44]. Thus, the use of hormonal protocols associated with artificial insemination is a viable alternative to facilitate the management of these females, since it eliminates the need for estrus detection and also increases reproductive rates, even in periods of seasonal anestrus [96,98,99]. These protocols are important for the efficient production of milk throughout the year, considering the demand of the consumers [106,107].

Prostaglandin (PGF2α) is a hormone used to control the estrous cycle, which aims to induce luteolysis and regression of the corpus luteum leading to the end of an estrous cycle [108]. In this way, it is used for the induction of estrus in buffaloes, improving reproductive efficiency and decreasing the interval between calving [97,109]. To reduce the variability of ovulation after the use of prostaglandin, GnRH has been used to control the follicular dynamics in this species [110,111].

The conception rates in buffaloes using artificial insemination with frozen semen is quite variable. In Surti buffaloes these rates have varied between 37.5% and 59.1% using insemination with frozen semen in different diluents [55,112]. Villa and Fabbri [113] suggested, from studies with Italian buffaloes, that the high variation in conception rates (30.5 to 57.1%) using frozen semen containing $8x10^6$ live spermatozoa is due to the strong influence of environmental factors.

Zicarelli et al. [114] hypothesized that the inferior results of AI in buffalo, when compared to cattle, occur due to the small size of the uterine body of the female buffalo compared to the cows, leading to the inadequate deposition of semen in only one of the uterine horns. According to Vale [10], a pregnancy rate of more than 50% may be considered good after insemination with frozen-thawed buffalo semen. Two inseminations during estrus is considered a good strategy to improve conception rates in buffaloes [115].

Fixed-Time Artificial Insemination (FTAI)

Fixed-time artificial insemination is a reproductive biotechnology to synchronize females, regardless of the phase of the estrous cycle they are in, followed by an insemination done at a fixed time, facilitating the farm management [99]. This biotechnology allows the improvement of reproductive indexes, shortening the interval between calving and conception, standardization of calf lots, and the use of proven bulls to increase herd quality. It also facilitates reproductive management, dispensing estrus observation, previously used for conventional artificial insemination [116,117].

In the buffalo species, this biotechnology is highly indicated due to its reproductive seasonality. There are periods when buffaloes present ovulation failure and absence of estrus manifestation [98], causing a decrease in the reproductive performance, increasing the interval between two consecutive pregnancies and the interval between births [38].

The Ovsynch protocol for FTAI was adapted to buffaloes by Baruselli [118] and basically consists of the initial administration of GnRH (D0), followed by a dose of prostaglandin seven days later (D7) and a second dose of GnRH after 48 hours (D9). Insemination is usually performed 16 hours after application of the second dose of GnRH (Figure 7). Using this protocol, Baruselli [11] reported conception rates ranging from 37.9 to 56.7%. Several factors may interfere with the results of this protocol, such as body score, period of the year, postpartum period and parturition order. Crudeli et al. [119]

evaluated the Ovsynch protocol in extensively raised buffaloes and obtained a 53% pregnancy rate.

Due to buffalo seasonality, FTAI protocols were created for the favorable and unfavorable reproductive season, which comprises the seasons of autumn and winter, and spring and summer respectively [96].

During the out-of-season period, there is often a high incidence of anestrus, which extends the interval between childbirth and a new conception, decreasing the productive indexes. Considering that exposure of females to progesterone may stimulates the development and maturation of the dominant follicle by increasing the release of LH, stimulating the development of the LH receptors and estradiol secretion, leading to ovulation, FTAI protocols using progesterone implants associated or not with exogenous estrogen were also tested. Fertility results for this type of protocol are very variable, ranging between 20% to 50% conception rates [98].

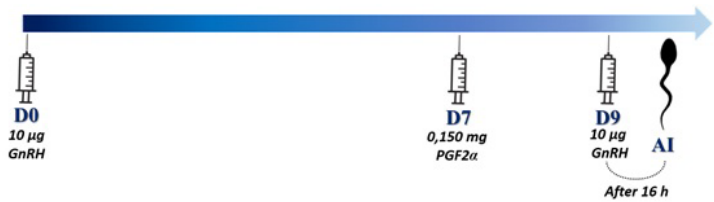

Figure 7: Ovsynch protocol, commonly used for FTAI during autumn and winter seasons (favorable reproductive seasons).

Buffalo females submitted to this protocol at the unfavorable breeding season (spring and summer) presented conception rates of 6.9%, when compared to the group submitted to insemination at the favorable reproductive season (autumn and winter) with 48.8% conception [96,99]. In Egypt, similar result was obtained, reinforcing the idea that the Ovsynch protocol is not ideal for the unfavorable reproductive seasons in buffaloes [120].

The hormone LH and hCG act directly in the ovulation process, while GnRH (gonadotrophin releasing hormone) acts at the pituitary stimulating the endogenous release of LH. Thus, the lack of endogenous LH causes failure in ovulation and those failures are more often during spring and summer [96]. Due to this characteristic of reproductive seasonality, alternative protocols were developed to improve the low conception rates during unfavorable reproductive seasons [121,122], promoting a distribution of births throughout the year, favoring the demands of the consumer for milk and dairy products [96].

Several protocols that used intravaginal progesterone implants during the unfavorable reproductive season showed low conception rates [114,123]. In this sense, Baruselli et al. [124] proposed a new protocol for buffaloes in seasonal anestrus and compared it with the conventional Ovsynch protocol. In the new protocol, on the first day (D0) 2 mg of estradiol benzoate was administered and the intravaginal progesterone implant was placed; on day 9, implant removal and administration of 0.150 mg PGF2α and 500 IU eCG were performed; on day 11, 1,500 IU of hCG was given and after 14 hours the insemination was performed. This protocol obtained higher conception rates (53.5%) than the conventional Ovsynch protocol (28.2%), proving to be a good option for the synchronization of ovulation and FTAI of buffalo females in an unfavorable reproductive season [96].

Subsequently, Baruselli et al. [125,126] demonstrated that eCG doses could be reduced to 400 IU and hCG to 1,000 IU without impairing conception rates, promoting a reduction in the cost of the protocol initially proposed for females in unfavorable reproductive seasons (Figure 8).

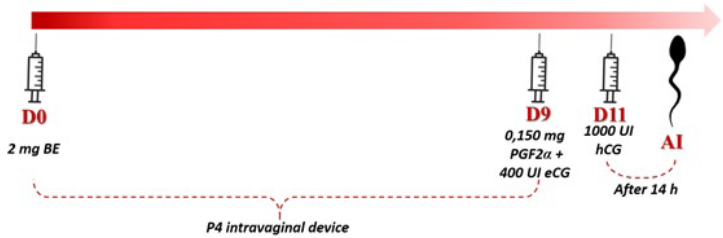

Figure 8: Protocol of FTAI proposed to be used during spring and summer (unfavorable reproductive seasons).

Conclusion

There are very noticeable differences in the reproductive characteristics and consequently in the results obtained with the use of biotechnologies in buffaloes, compared to other ruminants. For this reason, specific studies are being developed aiming to make feasible assisted reproductive techniques in these species, thus improving the productivity of buffalo herds to meet the increased demand for products offered by this species.

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