Chapter 04

Hormonal Applications in Horse Reproduction

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First Published June 11, 2018

This Book Chapter is an excerpt from an article published by Nisar Ahmad Chowdri at Clinics in Surgery in December 2016. (Chowdri NA. Ulcerative Colitis and the Surgeon. Clin Surg. 2016; 1: 1280.)

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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 estrogentic 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 biosynthetic 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), they 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 hypothalamo-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 hypothalamo-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, stanzolol 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 adenohypophysis 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 pre-cise signs of endometritis and may be led by a failure in uterine con-tractility [54], which explains why ecbolic agents are widely used in assisted reproduction programs in mares. The uterine contents are re moved 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 con tractions [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 myoe lectrical 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 uter ine 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 consist ent uterine response [59]. Compared to oxytocin, cloprostenol stimu lates 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 antagonists, 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 intra-vaginal 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 F₂α 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.

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

* 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-inflammatory 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 enable 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.

Acknowledgment

The author thanks FAPESP for the financial support, UNESP campus Botucatu, SP, Brazil for providing mares and structural space and Dr. Meira for the accomplishment of many experiments here described.

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