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MANIPULATION OF THE MARE'S ESTROUS CYCLE FOR FIXED-TIME ARTIFICIAL INSEMINATION (TAI)

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ABSTRACT

The application of modern biotechnologies in equine reproduction, including embryo transfer and artificial insemination, requires strict control of the estrous cycle. The peculiarities of the reproductive physiology of horses, such as seasonal reproductive activity and the prolonged and very variable duration of heat in cyclic mares, make it very important to detect the optimal moment of mating or insemination. In the cow, the optimal moment of insemination can be observed with some ease, due to the short duration of heat, assuming its correct observation. In the equine such procedure is not possible, that is, the mare usually needs to be mated more than once during heat or the coverage/insemination has to be determined by the veterinarian through transrectal follicular control, so the present work aims to show the evolution of pharmacological treatments and clarify at what stage the biotechnological tool known as fixed-time artificial insemination (TAI) is.

Keywords: Biotechnology. Equine. Reproduction. Artificial insemination.

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INTRODUCTION

About horses, the implementation of a fixed-time artificial insemination (TAI) program must take several particularities into account, one of them is the biotechnological processing of the semen to be deposited in the mare's uterus, due to the marked difference in the survival of spermatozoa submitted to different processing in the genital tract of this species. Currently, the deposition of semen in the genital tract of mares to be impregnated is by artificial insemination (AI) with refrigerated/transported or frozen semen, so AIs must occur 24 hours before to 6 hours after ovulation for refrigerated semen and for frozen semen 8 hours before to 6 hours after ovulation. In the case of using fresh semen, AI can occur 48 hours before to 6 hours after ovulation. It can be inferred, therefore, that the survival of frozen/thawed spermatozoa is 8 to 12 hours, that of cooled/transported semen is 24 hours, and that of native/fresh semen is 48 hours.

Studies have shown reduced sperm reserves in the oviducts of mares inseminated with frozen semen (Reger *et al*., 2003), and there is evidence that cryopreservation can permanently induce capacitation-like changes and shorten sperm survival in the mare's reproductive tract (Watson *et al*., 2001).

To define the number of mares to participate in the FTAI program, the number of inseminating doses available at the time of insemination of the equine herd must be taken into account according to the type of semen that is being used. If it is frozen semen we can have many doses available at the time of insemination, with chilled semen the number of doses is much more limited, since its storage time with little decrease in fertility is 48 h and a normal size testicle produces 6 to 8 x 10^9 sptz/ejaculate which allows several 12 to 16 inseminating doses with 500x10^6 Sperm. Native semen follows the same calculations as refrigerated semen, with the only difference being that collection and dilution must be done at the time of insemination.

One of the important reproductive aspects to succeed in the FTAI of the equine herd is the synchrony of the dominant pre-ovulatory follicles ≥35 mm in diameter with a high degree of uterine edema between the mares, because, at this moment, the use of an ovulation inducer is very effective, causing 80 to 90% of the mares to ovulate in a short period, after drug administration, between 36 and 42 hours. At this point, it is possible to distribute the inseminations, taking into account the survival of the gametes. In the case of only one insemination, it is recommended to deposition the semen 30 hours after induction, so that the fertilization interval will be between 24 and 42 hours, that is, if ovulation occurs

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at 24 hours, this oocyte will be viable until 30 hours, since its survival is 6 hours, and when, At this point, at 30 h, the inseminating dose is deposited, from then on, any ovulation occurring in the next 12 hours will ensure the viable meeting of the oocyte with fertile spermatozoa. In the case of two inseminations, the fertilization interval increases a lot, and a first insemination is remarked at 24 hours and the second insemination at 40 hours, so that the range of probable fertilization will be from 6 pm to 52 hours.

Reger et al. (2003) described a clinical trial with mares being inseminated twice at 24 and 40 h after hCG injection with a pregnancy rate per cycle of 76.4% (26 pregnancies in 34 cycles) and a seasonal pregnancy rate of 86.6%. In a clinical trial in Italy, 26 of 34 mares conceived (76%) after two timed inseminations versus 15 of 21 (71%) conceived after a single insemination within 6 hours of ovulation (Loomis and Squires, 2005). In a study in Colorado, Reger *et al*. (2003), reported no difference in embryo retrieval rates for mares inseminated once within 6 h after ovulation with 800x 106 frozen and thawed sperm (60%) *versus* mares inseminated twice, at 24 and 40 h after application of deslorellin with $400x10^6$ total spermatozoa per insemination (55%).

Therefore, the use of AI with frozen semen greatly increases the importance of determining the timing and frequency of insemination about ovulation, since the survival of sperm in the mare's reproductive tract is much shorter than when using cooled, transported semen or fresh semen. The use of frozen semen in the insemination of mares markedly increases their management, in a way that contributes negatively to the dissemination of the technique, since it requires, in addition to the intense service of the veterinarian, a more refined technical skill of the veterinarian, which results in a marked increase in cost for the owner of the stud farm.

The use of frozen semen is not yet fully encouraged in some breeds, and there is still some skepticism about this. Some of the impediments to the widespread trade in equine frozen semen employment include higher costs, more intensive breeding management of mares, the need for experienced professionals for semen processing and monitoring the correct timing of semen deposition in the mare's reproductive tract, and fertility being lower than that of fresh or refrigerated semen for many stallions.

The peculiarities of the reproductive physiology of horses, such as seasonal reproductive activity and also the prolonged and very variable duration of heat in cyclic mares, makes it of great importance to detect the optimal moment of mating or insemination, differentiating in the cow where the optimal moment of insemination can be

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observed with some ease, due to the short duration of heat, assuming your correct observation. In horses, this procedure is not possible; that is, the mare usually needs to be mated more than once during heat, or the mating/insemination has to be determined by the veterinarian through transrectal follicular control.

In order to obtain a high pregnancy rate in equine practice and, in some cases, to interfere with the estrous cycle with medications, it is necessary to have an in-depth knowledge of the reproductive physiology of the mare, basically in the endocrinology and follicular dynamics of the sexual cycle of the mare. The knowledge about follicular dynamics and the use of different protocols to obtain an efficient exogenous control of it are in advanced stages of development in the bovine species, allowing an intimate relationship between the reproductive physiology of this species and the use of modern biotechnologies. In the equine species, the application of state-of-the-art technologies for better genetic use and greater reproductive efficiency still requires intense efforts due to its reproductive particularities.

The presumed reduced life expectancy of frozen and thawed stallion sperm in the mare's reproductive tract combined with the "per dose, no guarantee" system of semen marketing has led to the practice of three or four examinations a day in mares inseminated with frozen semen (Loomis and Squires, 2005). Then, ultrasound exams are performed every 6 hours to ensure the insemination of the semen at the critical moment. An alternative to this method is insemination at a fixed time after induction with a reliable ovulatory agent. Fixed-time insemination protocols have been instrumental in reducing the number of ultrasound exams required. A fixed-time protocol involves treatment with a reliable ovulatory agent and mares being inseminated at a fixed time after this treatment.

The objective of the present work is to show the evolution of pharmacological treatments, analyze their use in veterinary practice, and clarify at what stage the biotechnological tool known as fixed-time artificial insemination (TAI) in horses is located.

BACKGROUND

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The scarcity of studies related to fixed-time artificial insemination in horses interferes with the more widespread use of this biotechnological tool. With the dissemination of knowledge related to FTAI in horses, in the future, more efficient protocols can be developed that will be fundamental to reducing the number of ultrasound exams required. The literature also brings its share of new information about the FTAI technique, its impacts, benefits, and difficulties of use given the various particularities of the reproductive physiology of the broodmare.

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OBJECTIVES

To compile information from the literature on fixed-time artificial insemination (TAI) in horses, addressing the physiological particularities of this species in order to describe the protocols already developed, showing the state of the art that this reproductive intervention presents today.

METHODS

A bibliographic survey was carried out in the main databases (PubMed, SciELO, CAPES Journal, Web of Science), in the search tool scientific works from the years 1966 to 2021 were selected, some keywords used in the search were *"equine reproduction, Fixed-time artificial insemination, mares, PGF2-α, progesterone, estrogen,* follicular dynamics*".,* 62 scientific works were selected.

THEORETICAL FRAMEWORK

USE OF PROSTAGLANDINS

Among the prostaglandins, prostaglandin F2α (PGF2α) and its analogues are the most commonly used hormones in equine reproduction. It presents an exceptional contribution when used alone to induce heat in cyclic mares or when in support of the use of biotechniques such as artificial insemination and embryo transfer (Faria; Gadela, 2010).

Prostaglandin F2α, or one of its analogues, is a successful pharmacological instrument in the manipulation of the mare's estrous cycle, anticipating heat and, consequently, ovulation. PGF2α is considered the primary luteolytic agent in mares because, in non-pregnant females, it controls the lysis of the corpus luteum (CL) that occurs after its release by endometrial cells between days 13 and 16 after ovulation (Milvae *et al*., 1996). In the natural cycle, in the absence of a pregnancy, prostaglandin is produced at a set time (12-14 days) after ovulation. As such, promoting the end of the luteal phase triggers with this, the beginning of endogenous hormonal changes associated with estrus and ovulation (Allen; Rowson, 1973; Loy *et al*., 1979; Neely *et al*., 1979; Cooper, 1981; Savage; Liptrap, 1987).

It can be used to end a persistent luteal or lactational anestrus phase, control ovulation time, induce gonadotropin secretion, synchronize estrus, treat mares with endometritis, eliminate pseudopregnancy (Mckinnon; Voss, 1992), stimulate uterine contraction, induce labor (Rossdale *et al*., 1979; Ousey *et al*., 1984) and promote

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miscarriages before the formation of endometrial calyces (35-40 days) (Neely, 1983; Mckinnon; Voss, 1992). It also acts in sperm transport, tubal motility, and contraction of the vas deferens (Hafez; Hafez, 2004). The administration of exogenous prostaglandin, as long as it is not within certain moments of the luteal phase period, allows its closure to be controlled, and with it, the manipulation of estrus and ovulation.

Although prostaglandin F2α can be administered intramuscularly (IM), intravenously (IV), intrauterine (IU), or intraluteally, the intramuscular route is preferred as it combines practicality with fewer side effects. These are observed in about 10% of mares within minutes of their administration. The most frequent signs are sweating, tachycardia, abdominal disorders, motor incoordination, and prostration (Lutalyse®, Pfizer Saúde Animal; manufacturer's package insert). In addition to being able to be applied by the means mentioned above, Alvarenga *et al*. (1998) tested the efficacy of the use of microdoses (1/10 of the minimum recommended dose) deposited in the Bai Hui acupoint (lumbosacral space) in mares during the luteal phase. They found the same luteolytic effect as when the conventional dose was administered intramuscularly. Return to estrus after application is observed in two to four days (Neely, 1983) or three to five days (Kotwica *et al*., 2002), and ovulation in seven to 12 days (Neely, 1983).

Due to its indirect effect on the release of GnRH and, consequently, of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), PGF2α can also be used to stimulate follicular growth and ovulation in transitional mares (Neely, 1983). They can be used in their natural form (prostaglandin F2α and dinoprostromethamine 5 to 10 mg i.m.) or as an analogue (α-prostol, 3 mg i.m.; fluprostenol, 0.25 µg i.m.; prostalene, 2 mg s.c.; or frenprostalene, 250 mg i.m.; coprostenol). The indicated doses are those suitable for a mare weighing an average of 400-500 kg (Le Blanc, 1995).

The success of prostaglandin in synchronizing estrus in the mare is variable and depends on the phase of the cycle in which she is. The corpus luteum of most mares is refractory to prior prostaglandin treatment until the 5th day of the estrous cycle, always counting day 0 (zero) as the day of ovulation (Douglas; Ginther, 1974; Loy *et al*., 1979). A good response is usually obtained when treatment is employed between the 6th and 9th days (Loy *et al*., 1979). To be successful, the treatment must not only end the luteal phase but also induce ovulation. There is considerable variation between the time of prostaglandin application and ovulation, with a variation of 24 h to 10 days being reported (Loy *et al*., 1979). The time interval is determined by the stage of follicular development

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during prostaglandin administration. Follicles up to 40 mm in diameter, or larger, ovulate on average within 6 days, although, again, considerable variation is reported. If the follicle ovularizes within 72 hours, it is often accompanied by an abbreviated estrus or absence of estrus. Occasionally, when a large follicle is present, prostaglandin treatment results in the regression of this follicle, subsequently developing another follicle that will ovulate, so there will be a long time interval between treatment and ovulation (more than 8 days) (Loy *et al*., 1979). The most consistent results are obtained when mares are treated at the beginning of the cycle, with follicles smaller than 40 mm in diameter, in which case there is a smaller variation between the application of prostaglandin and the ovulation interval, which is an average interval of 6 days (Table 1).

Table 1. Synchronization of estrus and ovulation in the mare, using a single prostaglandin injection, and its hypothetical A.I. schedule (Loy *et al*., 1979).

Time	Drug and event
Day 0	Ovulation
Day 7	Prostaglandin F2-∞
Day 9	Estrous Home
Day 11	Can Ovulation Occur - IA
Day 13	AI – Continue every 48 hours until estrus stops or ovulation is confirmed

Source: Loy *et al*., (1979).

A considerable variation in the mare's response can be observed.

As for the end of the luteal phase, fenprosthalene (250mg), a long-lasting prostaglandin, can be used in the mare at the beginning of estrus (up to 60 h from its beginning) to accelerate and synchronize ovulation. Ovulation is reported in 81% of mares in the first 48 hours of treatment, compared to 31% in untreated controls. This type of treatment can, therefore, be used to improve the success of fixed-time artificial insemination, performed at 48 hours after treatment with this prostaglandin (Savage; Liptrap, 1987).

The previously described use of prostaglandin depends on a single injection. The major disadvantage of this procedure is that in order to optimize success rates, the phase of the mare's estrous cycle must be known. In small stud farms, where mares are monitored individually, this may not pose any problems. However, if A.I. is to be used in large groups of mares kept in a herd situation or in mares whose stage of the cycle is unknown, double injection of prostaglandin is necessary (Hyland; Bristol, 1979). Double

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administration of prostaglandin needs to be applied with an interval of 14 to 18 days (Table 2).

Table 2. Synchronization of estrus and ovulation in the mare, employing double administration of prostaglandin and its hypothetical A.I. schedule (Hyland; Bristol, 1979).

Time	Drug and event
Day 0	Prostaglandin F2-∞
Day 16	Prostaglandin F2-∞
Day 20	Estrous Home
Day 22	Can Ovulation Occur – IA
Day 24	AI – Continue every 48 hours until estrus stops or ovulation is confirmed

Source: Hyland; Bristol (1979).

A considerable variation in the mare's response can be observed.

The time of estrus onset with this treatment is effective, and it has been reported that 60% of mares start estrus within four days of the second injection and 92% show estrus within 6 days (Hyland; Bristol, 1979; Voss *et al*., 1979; *Squires et al*., 1983; Squires, 1995).

However, the timing of the estrous cycle phase and the timing of ovulation are very variable. Ovulation can occur anytime between two and 12 days after the second injection. Although FTAI from the 4th day after the second injection, followed by three more inseminations at 48h intervals results in normal conception rates, multiple inseminations significantly increase costs (Bristol, 1993).

USE OF PROSTAGLANDINS AND HUMAN CHORIONIC GONADOTROPIN (HCG).

hCG has a similar action to LH, therefore, it has been used effectively to induce ovulation in mares, reducing the duration of estrus and the interval until ovulation (within 48 h), which leads to a lower number of inseminations and necessary mating per estrus (Bergefelt, 2000; Ley, 2006). The fact of synchronizing estrus and ovulation increases fertility rates (Oliveira and Souza, 2003), plasma concentrations of progesterone, and pregnancy rates. The use of hCG also improves the results of artificial insemination with refrigerated or frozen semen and embryo transfer (Melo, 2006). Its application, in addition, in mares with more than one pre-ovulatory follicle increases the possibility of double ovulations (Woods; Ginther, 1983).

The dose of hCG ranges from 1500 to 4000 IU (on average 2500 to 3000 IU), intramuscularly or intravenously (McKinnon; Voss, 1992), and the basic requirements for its

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application are the presence of a follicle >35 mm in diameter in the ovary (Bergfelt, 2000; Ley, 2006) and uterine edema rated 2 or 3 (scale 0 to 3, Ley, 2006).

The improvement of the previous protocol is the additional use of 1500-3700 IU of human chorionic gonadotropin (hCG), the human placental gonadotropin with luteinizing properties (LH), and follicle stimulating hormone (FSH) that reinforces and complements the natural release of gonadotropins, to increase follicular development and more specifically ovulation. Its use has the primary purpose of accelerating ovulation, consequently reducing the duration of estrus and, consequently, allowing only two inseminations to result in normal conception rates (Table 3) (Voss *et al*., 1975).

Table 3. Synchronization of estrus and ovulation in the mare, using two applications of prostaglandin and one application of hCG, and its hypothetical calendar for A.I. (Voss *et al*., 1975).

Time	Drug and event
Day 0	Prostaglandin F2-∞
Day 15	Prostaglandin F2-∞
Day 19	Estrous Home
Day 20	hCG
Day 21	Can Ovulation Occur – IA
Day 23	WOULD

Source: Voss *et al*., (1975).

A considerable variation in the mare's response can be observed.

Several times for the application of hCG have been postulated, most of them are between 4 and 6 days after the second application of prostaglandin (Palmer; Jousett, 1975; Douglas; Ginther, 1974; Hyland; Bristol, 1979; Voss *et al*., 1979; Bristol, 1981; Squire *et al*., 1983). Palmer (1976, 1979) reported that its use on day 6 after PGF2α application contributed to attenuating the problem of synchrony variability between ovulation and estrus. Palmer and Jousett (1975) reported that 75.8% of the mares ovulated within 72 hours of hCG application, which was administered 6 days after the administration of the second prostaglandin application. Yurdaydin *et al*. (1993) achieved similar success, using hCG 5 days after prostaglandin application, reporting that 80% of mares started estrus within 24-36h and ovulated 5-6.5 days after hCG administration. When used on day 8 after PGF2α application, estrus synchronization rates of 90% have been reported (Holtan *et al*., 1977). Other studies have shown a more variable reaction or no significant improvement with the use of hCG (Squires *et al*., 1983). It was recommended that the application of hCG be twice, once on day 7 (7 days after the 1st application of PGF2α) and once on day 21 (7 days after the 2nd PGF2α injection) (Table 4). The objective of this protocol is to

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encourage the development of a competent corpus luteum from the first prostaglandin injection, which will then react with less variation when the second prostaglandin application is made. This protocol resulted in up to 95% of mares ovulating on days 22 or 23 (Allen *et al*., 1974; Palmer; Jousett, 1975; Voss, 1993).

Table 4. Synchronization of estrus and ovulation in the mare, using two applications of prostaglandin and two of hCG, and its hypothetical calendar for A.I. (Allen *et al*., 1974; Palmer; Joussett, 1975; Voss, 1993).

Time	Drug and event
Day 0	Prostaglandin F2-∞
Day 7	hCG
Day 14	Prostaglandin F2-∞
Day 18	Estrous Home
Day 21	hCG
Day 22	Can Ovulation Occur – IA
Day 24	WOULD

Source: Allen *et al*., 1974; Palmer; Joussett, 1975; Voss, 1993.

It was found by Voss (1993) that the reaction of the follicle to the application of hCG depends on the phase of the breeding season. In the middle of the season, many mares ovulate spontaneously before the application of hCG is done on day 6 after prostaglandin administration. It is postulated that, during the peak of the reproductive season, the interval between the second application of prostaglandin and that of hCG should be reduced to less than 6 days so that a higher percentage of follicles react and ovulate within 48 hours. Although reasonably successful in inducing ovulation, hCG has one major drawback. Repeated administration makes the mare refractory to hCG due to the development of antibodies (Wilson *et al*., 1990), therefore, gonadotropin-releasing hormone (GnRH) and its analogues have been recommended for use instead. GnRH works by stimulating the natural release of LH and FSH from the anterior pituitary. As such, its administration in the form of a series of multiple injections (four at 12h intervals) or through subcutaneous implants has been shown to significantly induce the occurrence of ovulation in mares with follicles larger than 30 mm in diameter (Table 5).

USE OF PROGESTERONE

Progesterone supplementation and subsequent withdrawal can also be used for estrus and ovulation to occur. The use of progesterone, or one of its analogues, works on the principle of mimicking a mare's natural period of diestrus or the natural luteal phase. That is, simulating the natural production of progesterone during the luteal phase through the exogenous administration of progestins. The termination of this induced luteal state,

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through the cessation of treatment, acts as the end of the natural luteal phase and therefore induces changes in the mare's endogenous hormones, which are a prerequisite for the triggering of heat and ovulation (Handler *et al*., 2007).

Progesterone can be used in its natural form, in suspension in oil or propylene glycol, or as progestins, e.g., altrenogest (aliltrenbolone). It can be administered orally (0.044 mg altrenogest (regumate®; kg-¹ body weight/day) intramuscularly at a dose of 150-300 mg progesterone per day suspended in oil (Squires *et al*. 1983, Hughes; Loy, 1978), or, recently in long-acting progesterone ($P4^{LA}$) IM at a dose of 1,500 mg per week (Brigel *et al*. 2003), or via intravaginal sponges (impregnated with 0.5–1.0g of altrenogest inserted for 20 days (Palmer, 1985). Other researchers have investigated the use of PRIDs (intravaginal progesterone-releasing device) in mares, according to the methods available for cattle (Rutten *et al*., 1986) or CIDRs (controlled drug-releasing devices) containing 1.9 g of progesterone (Arbeiter *et al*., 1994). Whichever method of administration is chosen, treatment should be long enough (15–18 days) to ensure that any natural corpus luteum has time to regress before exogenous progesterone is withdrawn. The subsequent termination of the progesterone treatment will, therefore, be to remove the progesterone release device from the mare's body. Within 2 to 3 days of progesterone supplementation, the mare will normally cease all estrous activity, which will remain suppressed until the treatment is completed (Loy; Swann, 1966). After 15 days of treatment, estrous behavior presents within 3-7 days after removal of the device (Table 6) (Van Niekerk *et al*., 1973). Squires *et al*.. (1983) found similar results but indicated that ovulation synchronization was later, around 5.4 days on average. Progesterone or supplementation with analogous progestogen via intramuscular administration has been reported to show inconsistent results concerning ovulation synchronization (Loy and Swann, 1966; Squires *et al*., 1979). However, other researchers have reported that the rate of conception after 15 days of progesterone treatment is comparable to those associated with naturally occurring estrus (Van Niekerk *et al*., 1973; Squires *et al*., 1979b, 1983).

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Table 6. Synchronization of estrus and ovulation in the mare, with progesterone supplementation, and its hypothetical calendar for A.I. (Van Niekerk *et al*., 1973).

Time	Drug and event
Day 0 to 16	Progesterone supplementation
Day 19	Oestrus
Day 21	Estrous Home
Day 21	Ovulation – A.I.
Day 23	AI – continue every 48 hours until estrus stops or ovulation is confirmed

Source: Van Niekerk *et al*., (1973).

A considerable variation in the mare's response can be observed.

Traditionally, long periods of progesterone supplementation were used (up to 20 days), and although estrus was suppressed, ovulation was not necessarily suppressed. Thus, ovulation synchronization was not successful (Loy; Swann, 1966). Consequently, the use of hCG after cessation of progesterone supplementation was investigated, to enhance the synchrony of estrus and ovulation, as it appears to do when used with prostaglandins. However, only limited success has been reported (Holtan *et al*., 1977; Palmer, 1976, 1979). Currently, short periods of progesterone supplementation are being used. As the supplementation period may not be long enough to ensure that the natural corpus luteum has regressed, then, the treatment began to consist of progesterone supplementation combined with the use of prostaglandin (Silva *et al*., 2006). The use of progesterone has some drawbacks, mainly its association with reduced neutrophil production in response to a bacterial challenge, which can be very important in mares with poor perineal conformation or a history of uterine infections (Alexander *et al*., 1991).

USE OF PROSTAGLANDINS AND PROGESTERONE

There are several combination therapies, some of which have already been mentioned. The most commonly used and of current interest is progesterone with prostaglandin and progesterone with estradiol. Combined progesterone and prostaglandin therapies are becoming increasingly popular, as these treatments have often improved the prediction of the timing of ovulation and can reduce the duration of progesterone supplementation. Progesterone can be administered by one of several methods already discussed. It can be administered for long periods, over 20 days as reported by Draincourt and Palmer (1982) who administered 0.5g or 1.0g of altrenogest via intravaginal sponges for 20 days with PGF2-α administration on the day of sponge removal, resulting in estrus of

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1.8 (+/- 0.5) days and 2.2 (+/- 0.5) days, respectively, after PGF2α application and ovulation at 3.0 (+/- 0.7) and 5.4 (+/- 1.5) days, respectively, after PGF2α injection.

Today the administration of progesterone is usually done throughout only 7 to 9 days, with the application of prostaglandin on the day when the treatment with progesterone ceases. Using this same protocol, and again with intravaginal sponges containing 0.5g altrenogest, Palmer (1979) demonstrated that on average estrus occurred earlier (3.8 days) than the values suggested for progesterone treatment alone. The synchronization of ovulation was very variable, with values ranging from 8 to 15 days after prostaglandin application. A study carried out by Palmer *et al*. (1984), using the same sponges, but inserted for 7 days, with the application of 250 µg prostaglandin on the day of sponge removal, suggested a better synchronization of ovulation, which occurred in 10.1 – 14.0 days after sponge removal (Table 7).

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Table 7. Synchronization of estrus and ovulation in the mare, with progesterone supplementation and prostaglandin application, and its hypothetical schedule for A.I. (Palmer *et al*., 1984).

Time	Drug and event
Day 0 to 8	Progesterone supplementation
Day 8	Prostaglandin F2-∞
Day 12	Estrous Home
Day 16	Ovulation – A.I.
Day 18	AI – continue every 48 hours until estrus stops or ovulation is confirmed

Source: Palmer *et al*., (1984).

A considerable variation in the mare's response can be observed.

If the synchronization response in this treatment is evaluated about the different periods of the year, it becomes evident that, in April (northern hemisphere – early spring), the average time from sponge removal to ovulation was 14 days, but during the period from May to September (northern hemisphere – mid-spring and summer) the synchrony was very good. with ovulation occurring between 10 and 10.7 days. These results corroborate previous studies that also indicated a seasonal effect on the response of mares to progesterone and prostaglandin treatment (Draincourt; Palmer, 1982). It is evident that there is considerable variation in the response and that this variation is greater when using the protocol described above at the beginning of the physiological breeding season (Hughes; Loy, 1978).

USE OF PROSTAGLANDINS, PROGESTERONE, AND HUMAN CHORIONIC GONADOTROPIN (HCG)

Another alternative is the additional use of hCG to stimulate ovulation. It has been used after 6 days of progesterone supplementation and prostaglandin injection on the day progesterone was withdrawn (Table 8). This protocol resulted in ovulation rates of 52.3% and 75% at 48 hours and 96 hours, respectively (Palmer, 1979), and is also quite variable.

Table 8. Synchronization of estrus and ovulation in the mare, with progesterone supplementation and application of prostaglandin and hCG, and its hypothetical schedule for A.I. (Palmer *et al*., 1979).

Time	Drug and event
Day 0 to 8	Progesterone supplementation
Day 8	Prostaglandin F2-∞
Day 12	Estrous Home
Day 14	hCG
Day 16	Ovulation – A.I.
Day 18	AI – continue every 48 hours until estrus stops or ovulation is confirmed

Source: Palmer *et al*., (1979).

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A considerable variation in the mare's response can be seen

USE OF PROGESTERONE AND ESTRADIOL

This combination of hormones used as a biotechnological tool for FTAI in horses is increasingly popular. Both hormones can be administered daily IM for 10 days. Doses of 150 mg of progestogen and 10 mg of estradiol per day, followed, as in the previous protocols, by an injection of PGF2α at the end of treatment (Table 9), be successful, with a result of 81.3% of mares treated, ovulating 10-12 days after PGF2α injection (Loy *et al*., 1981). Normal pregnancy rates have been reported as a result of the A.I. of this treatment. PRIDs containing 2.3 mg progesterone and 10 mg estradiol (contained within a gelatin capsule) inserted for 10 days have been used with limited success (Rutten *et al*., 1986). There is a need for improvement of PRIDs or the development of subcutaneous capsules, with slow release, to increase the use of such hormonal combinations, eliminating the need for constant daily interventions.

Table 9. Synchronization of estrus and ovulation in the mare, with progesterone and estradiol, followed by the application of prostaglandin, and its hypothetical schedule for A.I. (Loy *et al*., 1981).

Time	Drug and event
Day 0 to 10	Progesterone and Estradiol
Day 10	Prostaglandin F2-∞
Day 20	Ovulation - A.I.
Day 22	AI – continue every 48 hours until estrus stops or ovulation is confirmed

Source: Loy *et al*., (1981).

A considerable variation in the mare's response can be seen

USE OF PROSTAGLANDINS AND GONADOTROPIN-RELEASING HORMONE (GNRH)

Buserelin acetate (Barrier-Battut, 2001), deslorellin acetate, GnRH agonists, in the form of short-acting implants (Hemberg *et al*., 2006) or in the BioRelease form (Fleury *et al*., 2003) and, more recently, fertirelin acetate (Santos *et al*., 2008), are efficient in increasing LH concentrations and inducing ovulation in cyclic mares (McKinnon *et al*., 1993; Mumford *et al*., 1995) and in a transitional period (McKinnon *et al*., 1997). However, the difference in ovulation time varies according to the drug used, being, on average, 24 to 48 hours for buserelin acetate (Barrier-Battut, 2001; Fleury *et al*., 2007) or 36 to 42 hours for deslorelin acetate (Samper *et al*., 2002), 36 to 48 hours for deslorelin BioRelease (Fleury *et al*., 2003) and 12 to 48 hours for fertirelin acetate (Santos *et al*., 2008). Recently, histrelin acetate has conferred satisfactory results in the induction and synchronization of

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ovulation at a dose of 0.25 mg (Kiser et al., 2013), Dallmann et al., (2021) verified a mean ovulation time for this drug of 21.8 ± 10 h and an interval of 12 hours to 36 hours.

The reduction in the number of dressings, as well as the number of veterinarian visits to perform follicular control when using deslorelin, makes it of great help for embryo transfer and artificial insemination programs, especially for refrigerated and frozen semen (Samper *et al*., 2002).

Table 5. Synchronization of estrus and ovulation in the mare, using two applications of prostaglandin and one application of GnRH, and its hypothetical calendar for A.I. (Meinert *et al*., 1993).

Time	Drug and event
Day 0	Prostaglandin F2-∞
Day 15	Prostaglandin F2-∞
Day 19	Estrous Home
Day 21	GnRH
Day 22	Can Ovulation Occur – IA
Day 24	WOULD

Source: Meinert *et al*., (1993).

A considerable variation in the mare's response can be seen

Rates of 88-100% of mares ovulating within 48 h of treatment with 1.5 to 2.25 mg of deslorelin have been reported (Meinert *et al*., 1993; Mumford *et al*., 1995). It has been suggested that GnRH may be more successful than hCG in inducing ovulation in larger, thicker-walled follicles. In addition, GnRH does not have the drawback of inducing response refractoriness due to antibody formation (Mumford et al., 1995), however, most of the work carried out to date on the use of GnRH has been in mares in natural estrus, rather than those submitted to protocols that synchronize estrus aiming at FTAI. The few studies on the use of GnRH with prostaglandin to induce estrus and ovulation suggest that there is no significant change in ovulation induction as a function of the treatment employed, compared to the use of prostaglandin alone (Squire *et al*., 1983). Therefore, although the regimen suggested in table 5 is likely to be feasible, it has yet to be proven to significantly improve the induction of estrus and ovulation.

Prostaglandin has some side effects, especially about its ability to activate smooth muscle contractions. Its use may be associated with increased gastrointestinal activity (manifesting as diarrhea), sweating, and possibly mild hind limb ataxia (Le Blanc, 1994). Side effects vary according to the drug used and the individuality of the mare and as long as the recommended dose is not exceeded, the effects are not serious.

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USE OF INTRAVAGINAL DEVICE WITH P4 (SEDP4), PGF2A AND HISTRELIN.

There is no indication by the manufacturing laboratories for the use of intravaginal devices in the equine species, only for the bovine, buffalo, caprine and sheep species.

Videla *et al..* (2002) conducted a study using intravaginal DIB devices (Syntex, S.A, Argentina) containing two different amounts of progestin; 1.38g and 1.90g. 2 groups of mares were used, each receiving devices with different concentrations of progestin. The devices remained for 12 days and during this period the animals had their ovarian activity monitored by ultrasonography and blood samples were also collected for progesterone dosages. At the end of the study, the authors concluded that, in order to obtain concentrations of serum progesterone sufficient to cause follicular growth inhibition and ovulation, the intravaginal devices used must contain at least 1.9 grams of progestin.

Wild *et al..* (2002) used PRID® in 11 mares for 12 days, and no manifestation of estrus was evidenced in any of the animals during this period. After the removal of the device, only one mare did not present estrus, and she had a history of anestrus of three years. Of the 10 mares that exhibited estrus, one (10%) manifested it 24 hours after removal, two (20%) after 18 hours and five after 72 hours. The remaining two presented estrus 5 to 6 days after removal of the device. The author attributed this dispersion to the fact that progesterone, despite having an efficient control over the manifestation of estrus, does not have an inhibitory effect on the secretion of FSH (follicle stimulating hormone), which is why there may be at the time of removal of the device a follicle in an advanced stage of development to the point of ovulating, which may enter atresia or ovulate immediately after removal of the device, without, however, there being an explicit manifestation of the signs of estrus by the mare. In other cases, the follicle present at the end of treatment may have a scarce development, causing the manifestation of estrus to be later (Squires *et al.*, 1979; Webel, 1975) taking into account the fact that one follicle grows per day during estrus. This led the authors to consider that PRID,2 a3 mm® although efficient in blocking the manifestation of estrus, presents considerable variations in the synchronization of ovulation. All the estrus presented by the 10 mares had normal manifestation, except for one that presented an estrus lasting 15 days. The mares received a dose of 2,500 IU of hCG (human chorionic gonadotropin) intravenously at the time they presented a follicle with a diameter greater than or equal to 35mm. Of the 11 mares treated, nine became pregnant, 8 through artificial insemination with refrigerated semen and one, with little pronounced estrus, through natural mounting, all performed 24 hours

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after hCG administration. The mare with prolonged estrus did not become pregnant. Despite the small number of animals used in this experiment, the authors considered that the pregnancy rate obtained was largely satisfactory and coincides with the results obtained by other authors (Van Niekerk *et al*., 1982) and suggest that the use of PRID® does not interfere with the fertility of mares.

Zielinsky (2020) proposed the protocol of intravaginal progesterone for nine days and administration of histrelin four days later (Table 10), proving to be effective in terms of the rate of embryonic retrieval after FTAI, stating that the intravaginal device with progesterone for nine days plus histrelin can be employed, dispensing with conventional daily follicular control.

Table 10. Synchronization of estrus and ovulation in the mare, with progesterone and estradiol, followed by prostaglandin application, and its hypothetical calendar for A.I., with embryo retrieval rate (ERT) (Zielinsky, 2020).

Time	Drug and event
Day 0 to 9	DIP4 and hCG (qdo. Fol>33 mm)
Day 9	Prostaglandin F2-∞
Day 13	Histrelin
Day 14	Ovulation - AI
Day 23	Embryo collection TRE= 29.41%

Legend: AI: artificial insemination; DIP4: Intravaginal device with controlled-release progesterone (1g P4, Biogénesis Bagó); PGF2α: D-Cloprostenol (75µg IM, Croniben, Biogenesis Bagó); Histrelin: histrelin acetate (500µg IM, Strelin, Botupharma, Botucatu, São Paulo); *hCG: Human Chorionic Gonadotropin (1250 IU IM, Vetecor, Hertape Calier) only in mares that had follicle >33mm, edema

Source: Zielinsky (2020) modified by the authors.

A considerable variation in the mare's response can be observed.

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