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Original

Synchronization of Follicular Wave and Ovulation Using Estradiol Benzoate and Progesterone in the Tropical-High Mares

Patricio Germán Cruz Oñate *, Juan José Zambrano *, Andrés Santiago Jácome Aucay **, Jorge Bolívar Dutan Sanango **, Luis Eduardo Ayala Guanga **

* The Technical University of Manabi, Faculty of Veterinary Sciences.
** Bachelor's Degree in Veterinary Medicine, Faculty of Agricultural Sciences, University of Cuenca, Ecuador.
Correspondence: luis.ayala@ucuenca.edu.ec

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ABSTRACT

Aim. To evaluate the effect of estradiol benzoate (EB) and an intravaginal progesterone implant to synchronize the estrus and ovulation of the tropical-high mares. **Methods:** A number of 24 creole mares were included in the study, distributed in two groups: T1=10 mg and T2 =25 mg of estradiol benzoate. The development of the dominant follicle (DF) was evaluated after the treatment, along with the levels of estradiol, every 48 hours, until a new follicular wave occurred. Later, subgroups were arranged according to the restart day of follicular wave occurrence during treatment 1 (T1_4d and T1_6d), and treatment 2 (T2_4d and T2_10d). **Results:** The mean DF size values in the two treatments (T1 and T2) were similar until day 4, then DF decreased gradually in T1, whereas T2 showed an increase until day 8, and the atresia process began. The restart of the follicular wave took place on the fourth day of treatment 1, and the fourth and tenth days of treatment 2. The time between the withdrawal of the implant and ovulation was similar in the subgroups: (T1_4d) treatment 1 and T2_4d, treatment 2. However, they differed from the other two subgroups observed in T1 and T2. **Conclusion:** The utilization of 10 mg estradiol benzoate along with a progesterone implant enabled better synchronization of the beginning of the follicular wave in a 4-6 post-treatment-day window (averaging 5 days).

Keywords: Sonography, follicles, follicular wave, ovaries, mare (*Source*: *MeSH*)

INTRODUCTION

Horses are a seasonal polyestrous, positively phototropic species. In countries with the four distinct seasons, reproduction occurs in early spring and throughout the summer (Cintora, 2005). However, in latitudes near the equator, there is little seasonal variation in terms of light hours,

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and the mares are considered an annual polyestrous species, so they can mate at any time of the year (Ramírez *et al.*, 2010). Therefore, the control of the estrus cycle and ovulation in mares is a reproductive handling aspect that requires further studies (dos Reis *et al.*, 2020).

The pharmacological agents used today to control the estrus cycle of mares are the same used in bovines; these protocols are sometimes implemented identically without considering the existing differences between species. Thus, in the 1970s, luteinizing agents (prostaglandins PG) were used for individual control of the estrus cycle of mares, as they were easy to administer (Rosa *et al.*, 2022). However, in contrast to cows, mares have a short luteal phase with a single follicular wave, generally. Hence, the programs based on shorter prostaglandin cycles are more likely to fail, which is mainly linked to the size of the ovulating follicle when the PG luteinizing dose is administered (Andrade *et al.*, 2011).

Besides, the ever-growing utilization of artificial insemination in this species demands group pharmacological control of the sexual cycle. In this context, several protocols have been tested with the inclusion of progesterone or synthetic progestogens using several routes of administration, such as oral, intramuscular (Oliveira *et al.*, 2018), subcutaneous (Handler *et al.*, 2007), sponges, and intravaginal devices impregnated with progesterone (Macan *et al.*, 2021).

The strategy for progestogen use consists of lengthening the luteal phase intentionally for a period above the duration of a normal corpus luteum. The blocking of the hypothalamus-hypophysial axis performed by the exogen progestogen inhibits the release of the luteinizing hormone (LH) during the treatment and stops the occurrence of estrus behavior. Sudden termination of treatment produces a similar effect to luteolysis, driving most females to begin estrus, usually on the third day after implant withdrawal (Polasek *et al.*, 2017). However, a progestogen-only treatment does not inhibit the release of the follicle-stimulating hormone (Bollwein *et al.*, 2004) from the adenohypophysis, thus prolonging follicular development.

Hence, research has been done on the utilization of high initial concentrations of progesterone combined with estrogen (dos Reis *et al.*, 2020), which is absorbed through the vaginal mucosa, causing inhibitory effects on LH and FSH, respectively, whose practical effect is to cause atresia of the dominant follicles and the emergence of a new follicular wave that produces a viable pre-ovulating follicle (Segabinazzi *et al.*, 2021).

Accordingly, this paper aimed to evaluate the effect of estradiol benzoate (EB) and an intravaginal progesterone implant to synchronize estrus and ovulation of the tropical-high mares on the equator plane.

MATERIALS AND METHODS

Animals and farm

A total of 24 creole mares aged 4-8 years were used in the experiment; the animals were engaged in touristic activities, with a 3-3.5 body condition (1-5 scale) (Henneke *et al.*, 1983). They belonged to two farms of the province of Pichincha, 2 900 m above seawater, and 10-20 ºC.

Before the experiments, transrectal sonography was performed and determined as cycles. Mare nutrition was based on pastures, commercial feeds, and mineral salts. The study relied on the sanitary code standard for ground-dwelling animals, chapter 7.8 Use of animals for research and education, World Organization for Animal Health (OIE, 2016).

Experimental design

The mares were distributed in two treatments at random: T1 (n = 12), which received 10 mg Estradiol Benzoate (Grafoleón®; Life, Quito, Ecuador), intramuscularly (IM), and T2 (n = 12), with 25 mg Estradiol Benzoate. Additionally, the two treatments included a progesterone-releasing intravaginal device (Sincrogest®; 1 g progesterone; Ourofino, Quito, Ecuador), and a dose (125mcg) of sodium cloprostenol (Estrumate, MDS, Quito, Ecuador) on day 0.

The assessment of the dominant follicle (DF) upon the application of the treatments was done through transrectal sonography (Aloka, Prosoun 2, Japan), using a 7.5 MHz linear probe every 48 h (08:00), until a new group of 9 mm follicles was observed (recruiting phase). The size of the DF was determined through the width length/2 ratio. Besides, from day 0, and every 48 hours, blood was collected (10 ML) from the coccygeal vein (08:00) in tubes without anticoagulant. The samples were centrifuged at 3000 G for 20 min, and the supernatant serum was frozen at -20 °C until the assessment of estradiol response was performed (Steranti Research, UK). The hormonal determination was performed by immunoassay. The standard estradiol curve range varied between 5 and 400 pg/ML, with 5.2 pg/ML sensitivity, with an 8.1% intra-assay variation coefficient.

The time considered as the beginning of the new follicular wave was the day when a new group of 9 mm follicles was observed.

The progesterone implant was withdrawn when the pre-ovulating follicle (POF) of the new follicular wave reached 35 mm. Then, transrectal sonography was performed every 12 hours, to observe the moment in which the POF disappeared, considered the moment of ovulation.

Statistics

SPSS, version 25® was used for the statistical analysis. Data normality was checked through the Shapiro-Wilk test. The effect of treatments on the variable's behavior of the DF (size), estradiol levels, and the moment of follicular wave restart (normally distributed), were analyzed through the T-Student test. Then, the animal groups were arranged according to the restart of the follicular wave day 1 (T1_4d and T1_6d), and treatment 2 (T2_4d and T2_10d); their influence on variables DF behavior, POF size, implant removal day, and ovulation day, by analysis of variance; the Tukey test (5%) was performed for the comparison of means.

RESULTS AND DISCUSSION

The mean size of the DF on day 0 was 26.1 ± 2.73 mm (T1), similar to the 24.3 ± 3.54 mm; P>0.05 (T2). The mean DF size values in the two treatments were similar until day 4 (Figure 1, panel A), then the DF size decreased gradually in T1, whereas T2 showed an increase until day 8, and the atresia process began. These features made the restart of the follicular wave. occur at different treatment times. The restart of the follicular wave in T1 took place on day 5 (average), below T2 (P<0.05), which took place on day 8 (average) (Figure 1, panel B).

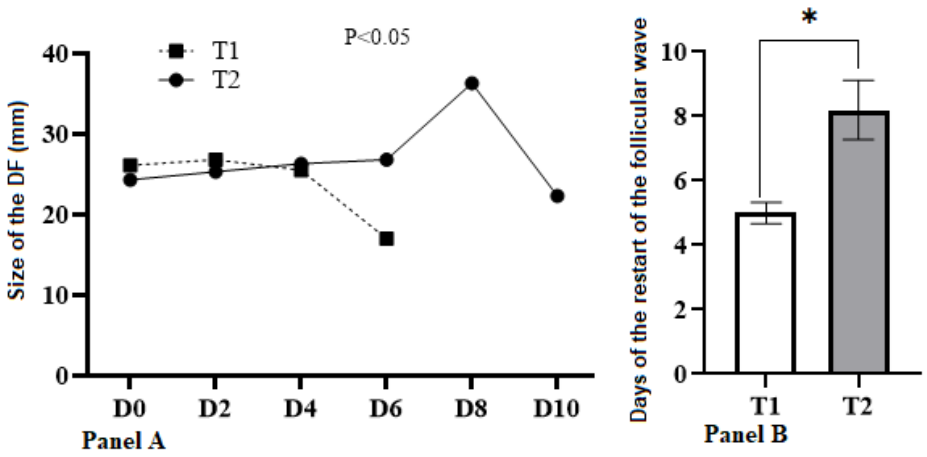

Figure 1. Behavior of the dominant follicle (DF) in the treatments T1=10 mg EB, and T2=25 mg EB, (Panel A) Mean daily values of the restart of the follicular wave in treatments 1 and 2 (Panel B).

The results of this paper confirm the existing scientific evidence about the use of estrogen-associated progesterone as an inhibitor of follicle development, by the suppressing action of the FSH secretion, which produces the restart of a new follicular wave in mares (Andrade *et al.*, 2011). However, progesterone alone does not have an inhibitory effect on FSH secretion; therefore, it must be combined with estrogens to produce such an effect (Segabinazzi *et al.*, 2021). This combination used in the study permitted the emergence of a new group of 9 mm follicles, which was considered the restarting day of the new follicular wave, as described by Andrade *et al.* (2011).

In an analysis of the estradiol concentration (E2) on day 0 before the treatment, similar levels were observed in T1 (54.5 1± 7.80 pg/ML) and T2 (68.8 ± 6.21 pg/ML; P>0.05). However, on the 2nd day (post-treatment), the T2 levels were higher (113.2 ± 6.93 pg/ML) than T1 (78.5 ± 11.50 pg/ML), with differences between the groups (P<0.05), just like on day 4 (Figure 2).

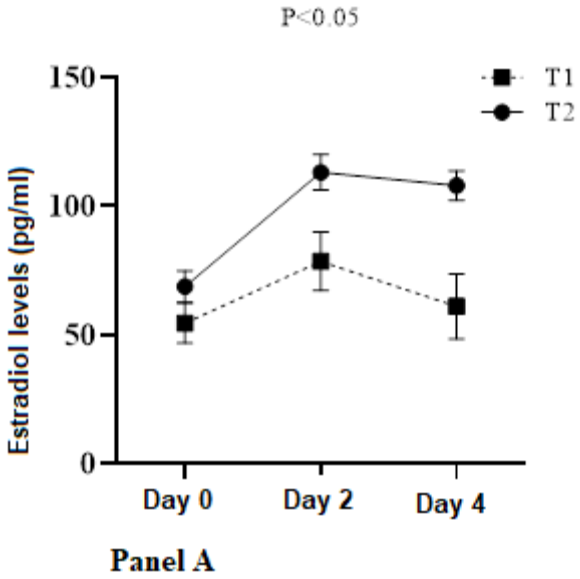

Figure 2. Estradiol levels in the treatments T1=10 mg EB and T2=25 mg EB, after the treatments The T-Student test

The higher estradiol concentration during the second treatment on days 2 and 4 of the experiment was linked to the EB dose administered on day 0 (25 mg), in T2. It was based on the product pharmacology, which describes that after EB intramuscular administration, it increases and keeps levels comparable to cyclic mares (Silva *et al.*, 2017).

Mean restart values of the follicular wave, size of the dominant follicle, and estrogen levels in treatment 1 and 2 subgroups

An analysis of the synchrony of the restart of the new follicular wave when administering EB and progesterone in the two treatments revealed that in T1 (n=10), the emergence of a new pool of 9 mm follicles took place in 50% of the mares (n=5), four and two days after the remaining five animals (day 6), accounting for 50%. These results can help determine a 48-hour window between the two groups (T1_4d and T1_6d) of the animals included in treatment 1. In treatment 2, the restart of the follicular wave occurred on the fourth day in three mares (30%; T2_4d), and the remaining seven mares on day 10 (70%; T2_10d), evidencing that the administration of 10 mg of EB conferred higher homogeneity at the restart of the follicular wave compared to the 25 mg EB treatment (Figure 3).

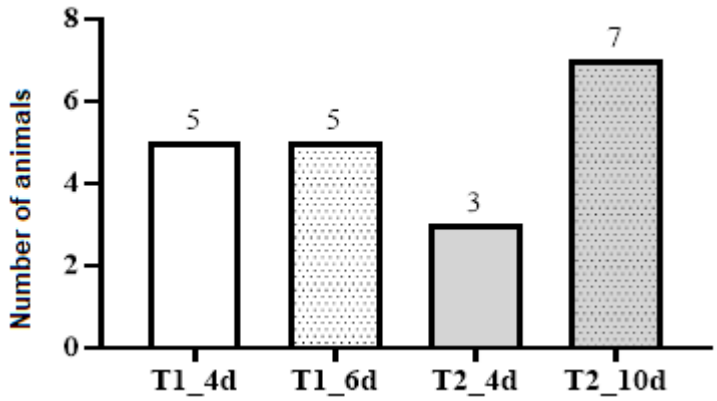

Figure 3. Number of animals that restarted the follicular wave on the fourth day of treatment (T1_4d) and 6 (T1_6d), and on the fourth (T2_4d) and tenth (T2_10d) days in treatment 2, after application.

The restart of the new follicular wave was closely associated with the DF size at the beginning of the protocol. Consequently, when the animal's DF was smaller than 25 mm and received 10 mg EB (T1_4d; 21.8 ± 3.18 mm), the DF atresia took place on the fourth day following the implementation of the protocol. However, when the DF reached ≥30 mm (T1_6d; 30.4 ± 3.74 mm), despite receiving 10 mg EB, atresia took place 48 hours later (day 6), compared to the T1_4d subgroup.

This peculiarity is associated with the fact that FD growth after selection (22 mm in mares) is less FSH-dependent, with a concentration kept at basal levels resulting from the production of estrogens and inhibin that causes negative feedback in the hypophysis, which permits minimum basal concentrations of essential FSH for the survival of DF (Andrade *et al.*, 2011).

The mares receiving 25 mg of EB (T2) had two subgroups T2_4d (24.0 ± 9.74 mm) and T2_10d (24.4 ± 3.65 mm); however, the time elapsed after the administration of EB to generate the DF atresia, was different, with a time window of 144 hours (6 days). Hence, the T2_4d mares were considered to respond to the synchronization protocol, whereas the T2_10d did not respond, and the wave restart took place upon the physiological atresia of the DF (Figure 4).

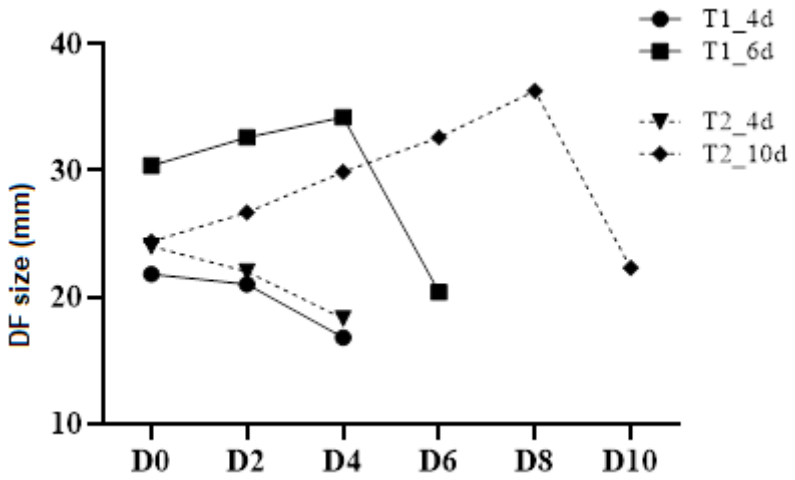

Figure 4. Behavior of the dominant follicle (DF) in the animals that restarted the follicular wave on days 4 (T1_4d) and 6 (T1_6d), and on days 4 (T2_4d) and 10 (T2_10d) in treatment 2, after application.

Behavior of the pre-ovulating follicle

The behavior of the pre-ovulating follicle (POF) was assessed depending on the restart day of the follicular wave. In the animals with a restart of the follicular wave at four days (T1_4d and T2_4d), the POF reached 35 mm or larger size, nine days after the restart of the follicular wave. However, in the groups in which the restart was 6 and 10 days after EB administration (T1_6d and T2_10d), the DF required more time to reach 35 mm (13 days on average) (Figure 5).

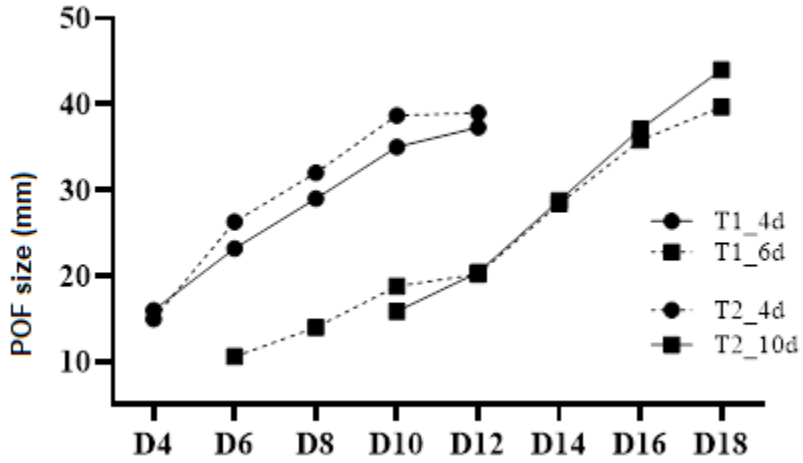

Figure 5. Behavior of the pre-ovulating follicle (POF), in the animals that restarted their follicular wave on days 4 (T1_4d) and 6 (T1_6d) in treatment 1, and days 4 (T2_4d) and 10 (T2_10d) on treatment 2.

Progesterone-Releasing implant withdrawal day

The progesterone implant was withdrawn when the POF reached 35 mm (Larocca *et al.*, 2006). In treatment 1 (T1_4d), at 11.2 ± 0.49 days and 16.4 ± 0.98 (T1_6d) days. In treatment 2 (T2_4d), at 10.0 ± 1.15 days and 15.7 ± 0.52 (T2_6d) days (Figure 6). The results of the implant withdrawal day in the T1_4d animals were similar to the T2_4d animals (P>0.05). However, both differed from the T1_6d and T2_10d (P<0.05), which, in turn, did not differ between them (P>0.05).

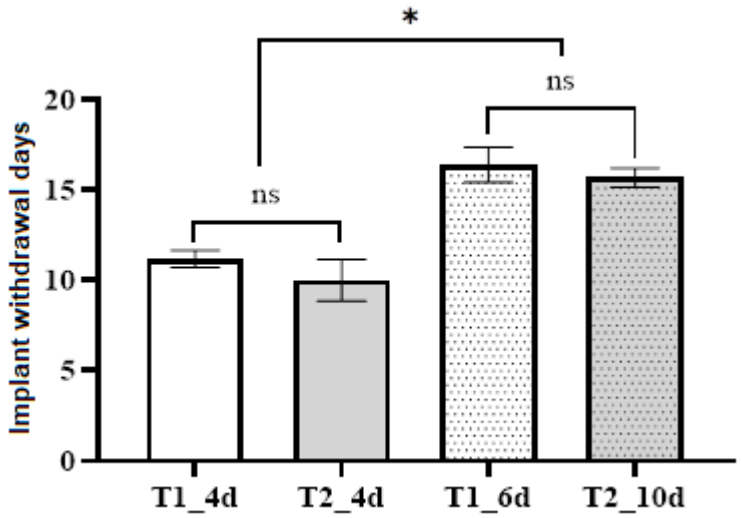

Figure 6. Progesterone-releasing implant withdrawal day from the animals that restarted the follicular wave on days 4 (T1_4d) and 6 (T1_6d) in treatment 1, and on days 4 (T2_4d) and 10 (T2_10d), in treatment 2.

The days needed for the withdrawal of the implant following the restart of the follicular wave were more than the ones described by Larocca *et al.* (2006), who organized the mares into two groups. In one group, an intravaginal device (1.38 g progesterone and 25 mg EB) was placed, and administration was IM; the other group received an implant (1.0 g progesterone and 25 mg EB IM). A 35 mm POF was reached after six to seven days, respectively. This difference may be associated with the amount of progesterone administered by Larocca *et al.* (2006).

Ovulating days and hours

This event took place 24-48 hours before ending estrus (dos Reis *et al.*, 2020), it occurred following an LH rise, which triggered a series of events that compromised the integrity of the ovarian tissue, with the interaction of vasoactive peptides, prostaglandins, and steroids as the main actors in the ovulating cascade. (Andrade *et al.*, 2011).

The ovulating day of T1_4d, occurred at 13.2 ± 0.49 days, and 19.2 ± 0.48 days (T1_6d), whereas in T2_4d, it was at 12.6 ± 0.67 days and at 19.1 ± 0.40 days (T2_10d) (Figure 7: Panel A). Regarding the ovulating hours, it occurred at 50.4 ± 2.40 hours (T1_4d) and 57.6 ± 0.98 hours (T1_6d); whereas for T2_4d, it occurred at 52.0 ± 0.0 hours and 63.4 ± 2.21 hours

(T2_10d) (Figure 7: Panel B). In the T1 animals that restarted the follicular wave at four and six days and the T2 animals that restarted their follicular wave at four days, no statistical differences were found. However, a comparison of these three groups with the T2_10 d showed differences at the ovulating times (P<0.05).

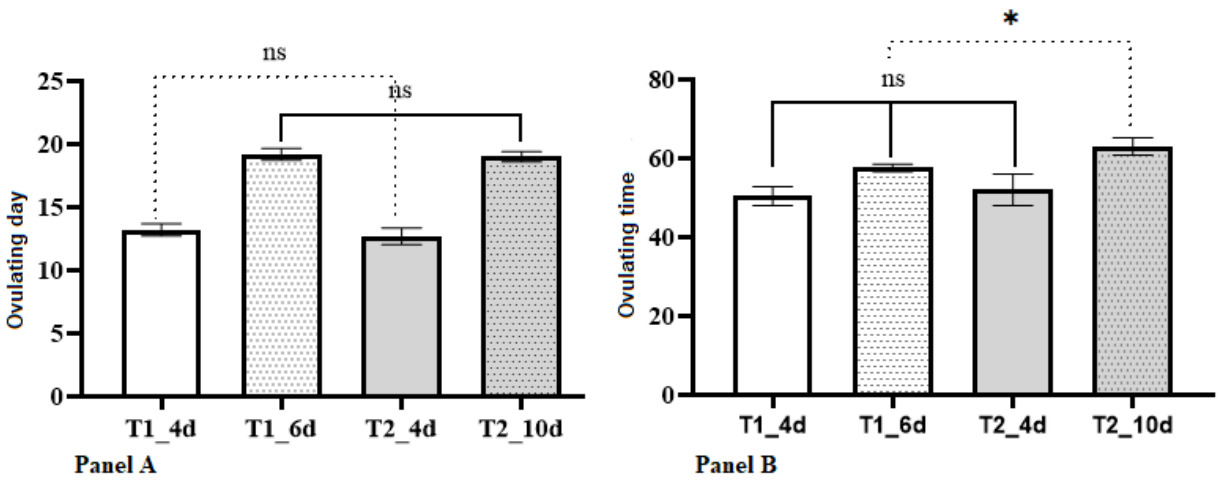

Figure 7. Ovulating day of animals that restarted their follicular wave on days 4 (T1_4d) and 6 (T1_6d) from treatment 1, and days 4 (T2_4d) and 10 (T2_10d) from treatment 2 (Panel A). Average ovulating hours of animals that restarted their follicular wave on days 4 (T1_4d) and 6 (T1_6d) from treatment 1, and days 4 (T2_4d) and 10 (T2_10d) from treatment 2 (Panel B).

In this study, the ovulating time took place 2.4 days after withdrawing the progesterone implant from the mares treated with 10 mg EB, which differed from the treatment using 25 mg EB, where the mean ovulating value was three days following the withdrawal of the progesterone implant (P>0.05). The values found in this study were below the values mentioned by Larocca *et al.* (2006), and the observations of Macan *et al.* (2021), who determined that ovulation takes place at 6.5 days following the withdrawal of the intravaginal device from mares.

CONCLUSIONS

The combination of a progesterone implant with a 10 mg estradiol benzoate dose improved estrus synchronization and ovulation in mares. However, higher EB doses (25 mg) reduced estrus efficiency, despite the existence of ovulation.

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AUTHOR CONTRIBUTION

Reseqarch conception and design: PGCO, JJZ, ASJA, JBDS, LEAG; data analysis and interpretation: PGCO, JJZ, ASJA, JBDS, LEAG; redaction of the manuscript: PGCO, JJZ, ASJA, JBDS, LEAG.

CONFLICT OF INTEREST

The author declares the existence of no conflicts of interests.