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Communication

[*Comunicação*]

Comparação descritiva de dosagens séricas de progesterona durante inserção de dispositivo intravaginal em éguas

[*Descriptive comparison of serum progesterone levels during intravaginal device insertion in mares*]

A.C.B. *Teixeira*[1], J.A.N. *Riveros*[1], I.C. *Pereira*[1], B. R. *Martins*[2], L.L. *Ledo*[2], M. F. *Brito*[3], G.A. *Monteiro*[4], F. P. *Leme*[4], L.Z. *Oliveira*[4]*

[1]Graduate, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[2] Undergraduate, Pontifícia Universidade Católica de Minas Gerais (PUC-MG), Belo Horizonte, MG, Brasil
[3]Pontifícia Universidade Católica de Minas Gerais (PUC-MG), Belo Horizonte, MG, Brasil
[4]Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

The equine industry plays an important role in the national and international agribusiness and the valuation of time savings and human resources is increasingly relevant in the field routine. Therefore, the use of hormonal protocols associating progesterone (P4) and estradiol (E2) to synchronize estrus and/or reduce the length of transitional periods are increasingly applicable in equine breeding systems.

In athletic mares, P4 is frequently used to reduce estrus behavior because, when associated with estradiol-17b, it plays a role of suppressing follicular development (Pinto *et al.*, 2004). Additionally, P4-based protocols can promote estrus synchronization (Faria and Gradela, 2010; Fedorka *et al.*, 2019) and cyclicity anticipation, decreasing the transition period and thus increasing the length of breeding season (Ginther *et al.*, 2004; Satué and Gardón, 2013).

During P4 treatment, LH secretion is suppressed. Hence, the treatment with P4 inhibits estrus expression, interrupting the simultaneous follicular maturation and positively affecting the accumulation of LH in the pituitary. At the end of P4 treatment, a large secretion of LH is provided with formation of a dominant follicle with ovulatory capacity. Therefore, hormonal protocols with P4 supplementation can provide several favorable results when correctly applied and outside the period of deep anestrus (Handler *et al.*, 2006; Hanlon and Firth, 2012; Teixeira *et al.*, 2021).

Progestogens are synthetic compounds that act on P4 receptors and may belong to different molecular classes and be available in different ways (Piette, 2018). In general, oral and injectable synthetic progestins are commercially available, as well as intravaginal devices (Faria and Gradela, 2010). Intravaginal progestogens are advantageous because they are easily applied and do not require daily treatment and/or injections (Negretti *et al.*, 2018).

In cattle, intravaginal P4 devices are widely used and ensure ovulation blockade, even when the implant is reused (Oliveira *et al.*, 2019). This second use depends on the initial P4 concentration of the device and animal category, but it is presented as an alternative to reduce the cost of the protocol. However, in mares, no studies were found in the literature using second-use devices or describing the serum concentrations of P4 during the device insertion. Therefore, the present study aims to describe the serum levels of P4 in mares treated with one or two first-use (new) intravaginal devices and even with a second use (reused) device in a period of ten consecutive days and to compare the P4 concentration obtained throughout the experimental period of the three treatments.

*Corresponding author: leticiazoccolaro@yahoo.com.br; leticiazo@vet.ufmg.br
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The experiment was performed on an experimental farm located in Esmeraldas (tropical climate), in the state of Minas Gerais, Brazil (latitude 19°45'46" S, longitude 44°18'47" W). Females were kept on the same pasture of *Cynodon dactylon* with water ad libitum, during the month of September 2021 (CEUA UFMG 49/2021).

Three crossbred healthy mares, aged between 8 and 14 years and body condition score 3 (Carroll and Huntington, 1988) were selected for the study. A previous gynecological examination was performed for each mare to assure the absence of corpus luteum in the ovaries and the absence of vaginal discharge, endometrial edema, or uterine alterations.

The intravaginal P4 release device used in this study was the Reprosync (GlobalGen, São Paulo, Brazil) containing 2.0 g of progesterone. In all animals the P4 devices were maintained for 10 days after insertion (D0 to D10). Mare 1 received two new (first use) P4 devices (two devices containing 2g of P4 each), mare 2 received a new P4 device (one device containing 2g of P4) and mare 3 received reused (second use) P4 device (a 2g P4 device previously used in another adult mare, for 10 days, before the experiment).

Blood collections for serum P4 dosage were performed before insertion of the device (D0), and daily, during the 10 days the device was in use (D0 to D10), in the morning (9:00am) and in the period of afternoon (15:00 pm).

The devices were intravaginally inserted with a specific applicator for this purpose (GlobalGen, São Paulo, Brazil) previously cleaned with a 1:3000 CB30 TA® solution (Alkyl dimethyl benzyl ammonium chloride, Ourofino Saúde Animal, São Paulo, Brazil) for 5 minutes. Additionally, the antibiotic oxytetracycline hydrochloride (Terra-Cotril® Spray, Zoetis, São Paulo, Brazil) was subsequently applied on the intravaginal applicator surface.

In the case of the used implant (second-use implant), after it was used for the first time, it was washed in a bucket with water and soap to remove dirtiness (after its removal from the first use) and then it was washed with running water. Subsequently, the device was cleaned with a 1:3000 CB30 TA® solution (Alkyl dimethyl benzyl ammonium chloride, Ourofino Saúde Animal, São Paulo, Brazil) for 5 minutes. After drying in the shade and at room temperature, it was stored in its original packaging until reuse.

Blood samples were obtained using siliconized tubes (red cap) and a 25mm x 8mm needle (Vacutainer® system), reserved at room temperature for 1 hour to clot. Then, the tubes were centrifuged (FANEM, model excelsa baby) for 15 minutes at 900x*g* at 20°C. The aliquoted serum was stored in polystyrene tubes at -20°C until analyzed.

Progesterone concentrations were analyzed by using the chemiluminescence method (IMMULITE 2000 XPi immunoassay system; Siemens Healthineers, Erlangen, Germany, USA) as described by Fazio *et al.* (2017) using a sequential competitive immunoassay (IMMULITE 2000 P4). The volume of 50 mL of serum was required for every cycle of incubation (2 x 30 minutes). The coefficient of variation for intra- and inter-assay were 6.3% and 7.9%, respectively (Teixeira *et al.,* 2020).

In the present work, serum P4 concentrations of mares receiving different amounts of intravaginal P4 were described. The intravaginal P4 devices have been used in horses because it is a non-invasive and practical procedure that allows rapid and prolonged absorption of P4 (Pohl *et al.*, 2009; Negretti *et al.,* 2018). Nevertheless, the commercially available devices were designed for the bovine anatomy which may cause losses of these devices when used in mares, or even undesirable local inflammatory reactions (Polasek *et al.,* 2017. In the present study, however, the P4-releasing intravaginal device used (commercially indicated for cattle) was easy to apply in the mares and no occurrences of expulsion or losses were observed during the days of the experiment. Additionally, in the mares of the present study, no vaginal discharge was identified.

Hence, considering that the use of intravaginal devices can lead to inflammatory process of vaginal mucosa due to the mechanical action they exert in the organ (Faria and Gradela, 2010; Polasek *et al.,* 2017, the importance of impregnating the devices with antibiotics prior to insertion is highlighted, as well as the correct

hygiene of the second-use device, to minimize the chances of developing vaginitis (Rutten *et al.,* 1986). In the study of Handler *et al.* (2006), which evaluated the use of intravaginal devices in mares, no previous use of antibiotics was performed and approximately one third of the animals presented moderate vaginitis. Still, according to other authors, when vaginitis occurs it is spontaneously resolved and does not have a negative impact on the total number of follicles, follicle diameter, ovulation rates, interval between P4 removal and ovulation, endometrial edema, or pregnancy rate, and does not affect embryo losses in recipient mares (Polasek *et al.,* 2017; Segabinazzi *et al.,* 2021).

In the present work, at the time that devices were inserted, none of the three mares had a corpus luteum (CL), which was confirmed by the serum hormone dosage showing P4 values below 1 ng/ml on D0 (am), as demonstrated in Table 1. From the insertion of intravaginal devices, serum P4 concentrations increased in all mares of the study and the values were already above 1ng/mL at six hours after the insertion (Table 1).

As previously demonstrated, regardless of seasonality, transvaginal absorption of P4 occurs immediately and effectively in the first hours after insertion (Handler *et al.,* 2006; Pohl *et al.,* 2009). The high absorptive capacity of vaginal mucosa and the type of material used for production of intravaginal implants allows this rapid hormone level rise (Handler *et al.,* 2006; Segabinazzi *et al.,* 2021).

The release mechanism of P4 from the device occurs by passive diffusion, and the hormone transfer is directly proportional to the magnitude of the concentration gradient across the membrane, the P4 liposolubility coefficient and the exposed surface area. In addition to the rapid hormone absorption right after the device insertion, this mechanism enables slow hormone release, maintenance of plasma levels for long periods (during the period of device presence) and sharp drop in P4 serum concentrations right after the device removal (Lavy *et al.,* 2006; Brunton and Parker, 2008).

Table 1. Descriptive values of serum progesterone (P4; ng/mL) concentration of mares receiving two new intravaginal P4 devices (2g of P4 each device), one new intravaginal P4 device (2g of P4) or used intravaginal P4 device (one previously used device of 2g of P4), inserted for 10 days

Intravaginal P4	D0 (am)	D0 (pm)	D2 (am)	D2 (pm)	D4 (am)	D4 (pm)	D6 (am)	D6 (pm)	D8 (am)	D8 (pm)	D10 (am)	D10 (pm)
Two new devices	0.60	1.50	8.89	8.44	5.30	4.21	4.37	4.56	3.49	4.49	4.11	1.76
One new device	0.48	4.21	5.51	3.61	3.32	3.32	2.96	2.56	2.25	3.36	2.43	0.91
One used device	0.41	3.36	4.00	3.21	2.23	2.70	2.78	3.20	2.30	3.08	1.09	0.51

D0 (am): Serum P4 concentration before device insertion (at 09:00 am of day 0); D0 (pm): Serum P4 concentration 6 hours after P4 insertion (at 15:00 of day 0); D2 (am): P4 concentration assessed in the morning of the second day of device presence (at 09:00 am of day 2); D2 (pm): P4 concentration assessed in the afternoon of the second day of device presence (at 15:00 of day 2); D4 (fourth day of device presence); D6 (sixth day of device presence); D8 (eighth day of device presence); D10 (am): P4 concentration assessed in the morning of the tenth day (last day) of the device insertion (at 09:00 am of day 10); D10 (pm): P4 concentration assessed 6 hours after P4 device removal (at 15:00 of day 10).

In Table 1 we also observe a sudden drop in the serum concentrations of the hormone in the 3 mares. However, in the mare that received two P4 devices (mare 1), the decrease in P4 concentrations occurred more slowly. At six hours after device removal, this animal still presented values above 1ng/mL due to the need for more time to metabolize the higher concentration of circulating P4 observed throughout the experimental period in this animal.

Similarly, Bisinotto *et al.* (2015) showed, in dairy cows, that after the device removal the serum P4 concentrations using two intravaginal devices decreases more slowly compared to one device (with values greater than 1ng/ml) and that the P4 levels of animals with two devices are closer to the hormone values produced by the CL compared to only one device. Also in dairy cows, Vasconcelos *et al.* (2018) showed that the use of a second device had a positive influence on follicular growth and oocyte quality compared to

animals with one device. Additionally, an improvement in the fertility of dairy cows was confirmed in the animals that ovulated larger follicles due to the increased circulating P4 promoted by the doble device (Vasconcelos *et al.*, 2018).

Considering the mean P4 levels throughout the entire experimental period for each animal (Table 2), it was observed that the mare that received two P4 implants (mare 1) had numerically higher mean serum P4 concentration than the mare that received one new P4 device or than the mare that received one used P4 device, which may be related to the larger contact surface of the two implants, compared with the contact surface with the vaginal mucosa of only one implant. Silva *et al.* (2021), using non-lactating Holstein cows, demonstrated that the size of surface area affects the ability of devices to release P4 since the greater the contact surface area, the greater the circulating concentrations of P4. Because the vaginal epithelium is highly permeable to steroids (and analogues), it is considered a fast and effective route of absorption for releasing the hormone into the circulation (Villanueva *et al.*, 1981; Rothen-Weinhold *et al.*, 2000; Corleta *et al.*, 2004).

On the other hand, it was interesting to note that the mares receiving one new device (mare 2), or a previously used device (mare 3), had relatively similar means of P4 levels, numerically, over the experimental period (Table 2). To the authors' knowledge, this is the first report to describe serum P4 concentrations in mares with a second-use intravaginal implant and it is also the first report to demonstrate that, in mares, the mean serum P4 concentrations seems to be similar in animals with new or reused intravaginal devices. On this basis, it may perhaps be speculated that, after the first use in mares, the levels of P4 impregnated in the device remained high enough to be reused in mares, and/or that the surfaces area in contact with the vaginal mucosa of both mares have the same limits of P4 absorption (by the vaginal mucosa), which prevents the animal that received a new implant from having more circulating P4.

Table 2. Descriptive means (± SD) of serum progesterone (P4; ng/mL) concentrations of mares receiving two (2g of P4 each device), one (2g of P4) or used (one previously used device of 2g of P4) intravaginal P4 device, inserted for 10 days

Intravaginal P4	D1	D3	D5	D9	Mean from D1 to D10
Two new devices	6.68±6.96	5.27±0.01	4.03±0.24	4.03±0.78	5.11±2.24
One new device	4.93±0.24	3.36±0.01	2.89±0.07	2.27±0.28	3.27±0.94
One used device	3.84±0.03	3.18±0.04	2.27±0.15	2.27±0.25	2.78±0.72

D1: mean of serum P4 concentrations assessed at morning and afternoon of the first day (day 1) of P4 device presence, of each mare; D3: mean of serum P4 concentrations assessed at morning and afternoon of the third day (day 3) of P4 device presence, of each mare; D5: mean of serum P4 concentrations assessed at morning and afternoon of the fifth day (day 5) of P4 device presence, of each mare; D9: mean of serum P4 concentrations assessed at morning and afternoon of the ninth day (day 9) of P4 device presence, of each mare; Mean from D1 to D10: overall mean of serum P4 concentrations throughout the entire experimental period of each mare (from the morning of day 1 until the morning of day 10; overall period of the presence of intravaginal P4 device).

Additionally, the Fig. 1 allows the graphic visualization of the numerical superiority of serum P4 levels in mare 1 (two P4 devices) at 24 hours after the implant insertion (D1) in relation to the other mares, reaching approximately 6.7 ng/ml of P4 in D1 and approximately 8.7 ng/ml of P4 in D2. As previously mentioned, this likely occurred due to the doubling of the initial P4 availability associated with the greater contact surface with the vaginal mucosa provided by the presence of two devices. Polo *et al.* (2016) demonstrated positive outcomes of the double administration of intravaginal P4 device in the induction of ovarian cyclicity in mares, during transitional and anestrus periods. The descriptive results of the present study also suggest that serum P4 concentrations can be different according to the number of devices inserted.

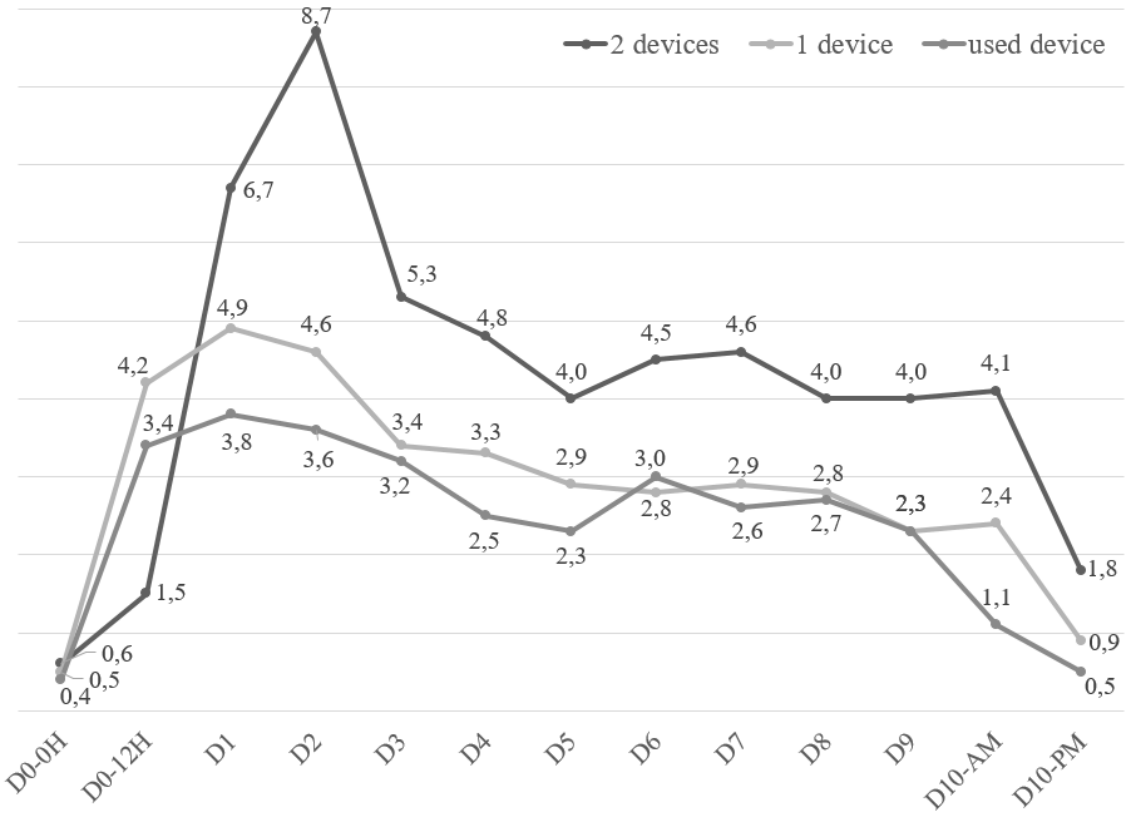

Figure 1. Serum progesterone (P4) concentration in mares with intravaginal P4 device; D0-0H: Serum progesterone (P4) concentration before device insertion; D0-12H: Serum P4 concentration 12 hours after P4 insertion; D1: serum P4 concentration at the first day (day 1) of device presence; D2 (pm): serum P4 concentration at the second day (day 2) of device presence; D10-AM: serum P4 concentration assessed during the morning of the tenth day (day 10) of the device insertion; D10-PM: P4 concentration assessed 12 hours after P4 device removal.

It is also noted in Fig. 1 that serum P4 levels remained relatively similar in mares that received one implant (new or used) throughout the experimental period. Additionally, until the end of P4 treatment (D10-AM), the serum P4 concentrations reached values above the minimum necessary to avoid ovulation, in the three mares of the experiment. According to Nett *et al.* (1976), a minimum circulating concentration of 2 ng/ml of P4 is needed so that ovulation does not occur during the period of permanence of P4 devices in mares. Because P4 exerts a negative feedback effect on the hypothalamic-pituitary axis and blockage of the cyclic LH release from the anterior pituitary, it suppresses estrus behavior of mares if plasma P4 concentrations exceed 1 to 2ng/mL (Aurich and Kaps, 2022). During the whole period of device permanence, serum P4 levels lower than 1 ng/ml were not observed in any of the three mares. Therefore, all treatments used in the present study (two new implants, one new implant or one used implant) seem to be possible options to prevent mares from ovulating while remaining intravaginally inserted, up to 10 days in the animals, although further studies (with a larger number of animals) are needed to confirm these findings.

Finally, as described above, it is important to emphasize the possibility of the maintenance of serum P4 concentrations, sufficient to maintain the ovulation blockade, with the use of a second-use implant. Considering the cost of hormonal protocols and the number of mares used in embryo transfer programs, the possibility of reusing the P4 devices may be an interesting alternative. However, future studies must be

carried out with a larger number of animals and further reproductive investigations to establish the correct conditions for reutilization of the devices without affecting reproductive rates.

Serum P4 concentrations of mares receiving different amounts of intravaginal P4 devices seem to differ according to the number of inserted devices. Still, all treatments (two new P4 devices, one new P4 device or one used P4 device) reached serum P4 concentrations compatible with promoting ovulation blockade. Hence, the use of used P4 devices may be recommended if further investigated in future studies.

Keywords**:** mare, ovulation blockade, P4 device, serum dosage

RESUMO

*O objetivo foi descrever os níveis séricos de progesterona (P4) de éguas tratadas com um ou dois dispositivos intravaginais de primeiro uso e com um dispositivo de segundo uso de P4, em um período de 10 dias e comparar a concentração de P4. Três éguas foram tratadas com implante intravaginal de P4 (Reprosync®; 2g de P4) por 10 dias (D0 a D10). A égua 1 recebeu dois implantes novos, a égua 2 recebeu um implante novo e a égua 3 recebeu um implante usado de P4. Coletas de sangue foram realizadas diariamente (*a.m./p.m.*) do D0 ao D10 e concentrações de P4 foram analisadas por quimioluminescência. A égua que recebeu dois dispositivos apresentou numericamente maior concentração sérica média de P4 (5,11±2,24ng/mL) do que a égua que recebeu um dispositivo novo (3,27±0,94) e do que a égua que recebeu um dispositivo usado (2,78±0,72). As éguas que receberam um dispositivo (novo ou usado) tiveram médias de P4 relativamente semelhantes numericamente. Concluiu-se que a concentração sérica de P4 de éguas recebendo diferentes quantidades de dispositivos intravaginais de P4 parece diferir de acordo com o número de implantes inseridos. Ainda assim, todos os tratamentos atingiram concentrações séricas de P4 capazes de bloquear a ovulação.*

Palavras-chave: bloqueio de ovulação, dispositivo de progesterona, dosagem sérica, éguas

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