Received: 20 May 2016 | Accepted: 17 July 2016

DOI: 10.1111/rda.12762

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

Hormonal changes and follicular activity after treatment with intravaginal progesterone-releasing devices in llamas

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Contents

Plasma progesterone (P_4) concentrations and follicular activity after administration of different P_4 doses were evaluated in 33 adult female llamas treated with intravaginal devices. In Study 1, a group of llamas (n = 10) was treated with an intravaginal device (IVD) containing 160 (n = 5) or 780 mg of P_4 (n = 5). Based on the results from the first study, in Study 2, females with follicles at different stages of development were treated with the IVD containing 780 mg of P_4 (n = 21) or remain untreated (control; n = 12) to evaluate the effect of P_4 on follicular activity. In Study 1, the IVD containing 160 mg of P_4 induced follicular turnover in 60% of females while the remaining 40% of llamas developed persistent follicles. Thus, this device controlled follicular activity in llamas, although it promotes the persistence of follicles present at start of treatment. Conversely, in both studies, the IVD containing 780 mg of P_4 suppressed follicular development and hasten the emergence of a new follicular wave in all females regardless of the follicular phase at insertion. Additionally, in Study 2, this device effectively concentrated the appearance of follicles with ovulatory diameter at a definite time after treatment in comparison with control animals. In conclusion, treatment with an IVD containing 780 mg of P_4 would be considered for the control of follicular activity in llamas as it ensures the presence of a young follicle with ovulatory diameter by day 6 after the end of treatment in all females.

1 | INTRODUCTION

Progesterone (P_4) is a steroid hormone synthesized and secreted by the corpus luteum (CL). In ruminant species, P_4 has a negative effect on follicular development through the inhibition of luteinizing hormone (LH) pulsatility (Adams, Matteri, & Ginther, 1992b; Adams, Matteri, Kastelic, Ko, & Ginther, 1992a; Seekallu, Toosi, & Rawlings, 2009; Sirois & Fortune, 1990). Thus, P_4 and progestagens are usually used to control follicular activity in ruminants and other species (*cows*: Adams et al., 1992b; *ewes*: Leyva, Buckrell, & Walton, 1998; *goats*: Menchaca & Rubianes, 2004; *mares*: Handler, Schönlieb, Hoppen, & Aurich, 2007). Previous studies have reported that P_4 treatment must induce luteal concentrations to suppress LH pulsatility and promote follicular turnover, inducing atresia of the dominant follicle and hastening the emergence of a new follicular wave (Adams et al., 1992a; Leyva et al., 1998; Sirois & Fortune, 1990). Conversely, subluteal levels of P_4 increase LH pulsatility and result in persistent growth of the dominant follicle and ovulation of follicles with aged oocytes, compromising fertility (Adams et al., 1992b; Austin, Mihm, Ryan, Williams, & Roche, 1999; Viñoles, Meikle, Forsberg, & Rubianes, 1999). The previous considerations emphasized the importance of selecting a treatment that induces luteal P_4 concentrations during the entire treatment.

Llamas (*Lama glama*) are induced ovulators. Thus, in non-mated females, follicular activity proceeds in the absence of a CL, with basal P_4 concentrations (<3.2 nmol/L; Bravo, Fowler, Stabenfeldt, & Lasley, 1990; Aba, 1995; Cavilla, Bianchi, Maistruarena, & Aba, 2013). Previous studies have reported that luteal P_4 concentrations inhibit follicular activity in llamas by reducing the number of follicles, oestradiol-17β (E_2) plasma concentrations and the diameter of the dominant follicle (Aba, 1995; Adams, Griffin, & Ginther, 1990).

The use of intravaginal devices (IVDs) and sponges in the implementation of progestin-based oestrous synchronization programs have

the advantage to involve less work than the repetitive administrations required by parenteral or oral applications. Moreover, IVDs could be reused successfully reducing the costs of treatments (Suarez et al., 2007; Vilariño, Rubianes, & Menchaca, 2011). In llamas, the use of an IVD containing 330 mg of P_4 (Chaves et al., 2002) or intravaginal sponges containing 120 mg of medroxyprogesterone acetate (MAP; Aba, Quiroga, Auza, Forsberg, & Kindahl, 1999; Aller, Cancino, Rebuffi, & Alberio, 2010) induced follicular regression regardless of the phase of the follicular wave at the start of treatment. In addition, intramuscular injections of P_4 suppressed follicular development (Santiani, Leyva, & García, 2002) and ensured the presence of a follicle deemed to ovulate at the end of treatment (Alberio & Aller, 1996; Carretero, Chaves, Agüero, & Miragaya, 2006). Besides, Aller et al. (2015) proved in superovulated llamas that all animals were sexually receptive by day 2 after the end of P_4 treatment, although no description of follicular development was reported in this study. Meanwhile, there are no studies characterizing plasma P_4 concentrations and their effects on follicular activity in llamas treated with IVDs containing lower or higher P_4 concentrations than those previously evaluated. Furthermore, a systematic characterization of the effect of P_4 on the regression pattern of follicles at different stages of development at insertion, on the emergence of a new follicle and its development until ovulatory size (≥7 mm) have not been included in previous studies in llamas.

The aims of this study were twofold: Firstly, to characterize follicular activity and plasma P_4 concentrations in llamas treated with two different intravaginal doses of P_4 using an IVD with a lower (160 mg) or higher (780 mg) P_4 content than those previously evaluated by other authors. Based on the results from the first study, the aim of the second study was to evaluate the effect of the intravaginal device containing 780 mg of P_4 on follicular activity considering the stage of the largest follicle (F1) at the beginning of treatment, on the emergence of the largest follicle (F2) of the successive wave and its development until ovulatory diameter of 7 mm.

2 | MATERIAL AND METHODS

2.1 | Animals

A total of 33 llamas were selected from a herd located at the Faculty of Veterinary Science, UNCPBA, Tandil, Argentina (37°S, 60°W), considering that they were non-pregnant, non-lactating, clinically healthy and with an adequate size for transrectal palpation. During the experimental period, females were kept separated from males and were fed pasture hay and had free access to water. The experimental design and animal care were performed in compliance with regulations set by the Animal Welfare Committee at the Faculty of Veterinary Sciences, UNCPBA, Tandil, Argentina.

2.2 | Treatments

2.2.1 | Study 1

Ten randomly selected female llamas were assigned into two groups: group 160 (n = 5) and group 780 (n = 5). Females of group 160 received an IVD containing 160 mg of P_4 (Cronipres co®, Biogénesis Bagó, Argentina), and females of group 780 received an IVD containing 780 mg of P_4 (a pod of the IVD Cue-mate®, Bioniche Animal Health, Australia). The IVD containing 160 mg is an intravaginal device designed for use in ovines and caprines while the IVD containing 780 mg was obtained through an adaptation of the IVD Cue-mate® designed for use in bovines (cows and heifers). The IVD Cue-mate® consists of two pods containing 780 mg of P_4 each embedded in a silicone matrix. In cows, both pods are mounted on a special device and the system is inserted intravaginally using an applicator provided by the manufacturer. In this study, only one pod was used for each treated llama female.

The IVD 160 was inserted using the applicator and instructions provided by the manufacturer. Conversely, for the IVD 780, a cord of approximately 40 cm was inserted through the holes of one of the pods making a knot near the side of the device, a special applicator was designed and the pod was mounted on the applicator allowing the cord to hang from it. All the system together was embedded in povidone iodine before their use. The applicator containing the IVD was inserted intravaginally at 10–15 cm depth. Once in position, the IVD was inserted into the anterior vagina and the applicator was retired. The IVDs were inserted into the vagina on day 0 of the study in all females and were withdrawn on day 7.

In *Study 1*, IVDs were inserted irrespective of the stage of ovarian follicular wave at start of treatment. Based on results of *Study 1*, the IVD 780 was selected to be used in *Study 2*. After a 1 month washout period, females from *Study 1* were used for *Study 2*.

2.2.2 | Study 2

Thirty-three female llamas were randomly assigned into two groups: progesterone-treated llamas (treated group; n = 21) and none-treated animals (control group; n = 12). In addition, each group was divided into three subgroups according to the phase of the follicular wave (growth, plateau and regression) of the largest follicle (F1) present at the beginning of the study (day 0). The phase of the follicle was determined in each female by transrectal ultrasonography during three consecutive days before the beginning of the study as previously reported by Cavilla et al. (2013). Thus, groups were defined as follows:

- *Group G*: Animals with a follicle at the *Growing* phase: follicles between 5 and 7 mm of diameter with two consecutive increasing measurements.
  - *Group Gc*: *Growing control* group (n = 4)
  - *Group Gt*: *Growing-treated* group (n = 7)
- *Group P*: Animals with a follicle at the *Plateau* phase: follicles that stabilized their diameter around the maximum (8–14 mm) with consecutive measurements oscillations <0.5 mm.
  - *Group Pc*: *Plateau control* group (n = 4)
  - *Group Pt*: *Plateau-treated* group (n = 7)

- *Group R*: Animals with a follicle at the *Regression* phase: follicles between 14 and 5 mm of diameter with two consecutive decreasing measurements.
  Group Rc: *Regression control* group (n = 4)
  Group Rt: *Regression-treated* group (n = 7)

The third ultrasonography, on which the phase of follicular wave was defined, was considered the day 0 of the study for each group. On day 0, in females of the progesterone-treated groups (*Gt*, *Pt y Rt* groups), the IVD containing 780 mg of P_4 was inserted into the vagina until device withdrawal on day 7. Conversely, females from the control groups (*Gc*, *Pc y Rc* groups) remained untreated.

2.3 | Definitions and ovarian ultrasonography

The largest follicle present at the beginning of the studies (day 0) was defined as F1. The largest follicle that originated from a new wave after day 0 of the study was defined as F2. The emergence of F2 was defined retrospectively when the F2 had a diameter of 3 mm. The mean age of the F2 present on day 7 of the study and when it reached the ovulatory size was determined considering the number of days elapsed from emergence as described for ewes (Viñoles et al., 1999) and goats (Menchaca, 2006). Ovulation was defined as the ultrasonographic disappearance of F2 (≥7 mm, Bravo, Stabenfeldt, Lasley, & Fowler, 1991) by 48 hr after buserelin (Receptal®, Intervet, Argentina) injection.

2.3.1 | Study 1

Transrectal ultrasonographies were performed daily in females from two days before device insertion (day 0 of the study) until a follicle with ovulatory size (≥7 mm) was detected after device withdrawal. During each ultrasonographic examination, the daily diameter of the largest follicle and the day of new wave emergence were registered as described previously.

2.3.2 | Study 2

Ultrasonographies were performed daily from two days before day 0 of the study (device insertion in treated animals) until a new follicle (F2) with ovulatory size was detected after day 7 of the study (device withdrawal in treated animals). During transrectal ultrasonographies, the effect of treatment on follicular dynamics, on the emergence of F2 and its development until ovulatory size were evaluated. Finally, when F2 reached a diameter ≥7 mm, ovulation was induced with an intravenous injection of 16.8 μg of buserelin (Receptal®, Intervet, Argentina). 48 hr after buserelin injection, ovulation was confirmed by ultrasonography.

2.4 | Blood sampling

Blood samples were collected into heparinized tubes. Venipuncture was performed alternately at high, medium and low positions on the left and right sides of the neck in order to minimize damage to the jugular veins due to the high frequency sampling protocol (Aba et al., 1999). Within 1 hr after extraction, plasma samples were separated by centrifugation and stored at −20°C until analysed.

In *Study 1* and in 16 selected treated females of *Study 2* (including females of the growing, plateau and regression subgroups), blood samples were collected immediately before insertion (hr 0), at hr 1, 3, 6, 12, 24 after insertion and then daily until day 8 after insertion (day 1 after withdrawal) to measure plasma P_4 concentrations. In control females of *Study 2*, blood samples were collected from day 0 to day 8 of the study. In addition, in six selected treated females of *Study 2* (including females of the growing, plateau and regression subgroups), blood samples were obtained daily from day 7 of the study (day of device withdrawal) until the F2 reached a diameter of 7 mm and in six control llamas when the F2 reached a diameter of 7 mm to measure oestradiol-17β (E_2) plasma concentrations. Finally, in 11 treated females of *Study 2*, ovulation of F2 was confirmed by P_4 measurements on day 4 and 8 after buserelin injection.

2.4.1 | Hormone determinations

Plasma P_4 concentrations were measured using a radioimmunoassay (RIA) Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) previously validated for use with llama plasma (Bianchi, Meikle, Sartore, Gonzalez, & Aba, 2007). The sensitivity of the assay was 0.3 nmol/L and the intra-assay coefficient of variation (CV) was below 13% for concentrations between 0.4 and 128 nmol/L. Oestradiol-17β (E_2) plasma concentrations were measured using a RIA Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) reported for use with bovine plasma (Sirois & Fortune, 1990) and validated for use with llama plasma after minor modifications (Aba, Forsberg, Kindhal, Sumar, & Edqvist, 1995). The intra-assay CV was below 11% for concentrations between 5.6 and 180 pmol/L. The lowest amount of E_2 detectable was 5.6 pmol/L. All samples were measured in duplicates and in a single assay for both hormones. Hormone concentrations are expressed in SI units. To convert from pmol L^{-1} to pg ml^{-1} and from nmol L^{-1} to ng ml^{-1}, a factor of 3.7 for E_2 and 3.2 for P_4 should be used.

2.5 | Data analysis

Results are expressed as mean ± S.E.M. Plasma P_4 concentrations from each group were analysed by *analysis of variance* (ANOVA) using a repeated measurements design (within-SS). Mean plasma P_4 concentrations and follicular diameter at each time were compared between groups using ANOVA followed by *Fisher's least significant difference test* (LSD) to determine differences between means. The area under the curve (AUC) for plasma concentrations vs time from time zero until the last concentration measured was calculated by the trapezoidal methods described by Baggot (1995). The AUC values obtained after insertion of both IVDs and the characteristics of the largest follicle present at device withdrawal were compared between groups by *Student t-test*. The differences between treated and control groups on

mean daily follicular diameter of F1 was analysed by ANOVA followed by *LSD test* to detect differences between means. Single data points as: mean diameter of F1 at day 0; day of F2 emergence; E_2 production and life span of F2 between control and treated groups were compared using *Student t-test*. In addition, the effect of treatment (control vs treated) and phase of the follicle at insertion (growing, plateau and regression) on emergence of F2 and the day on which that follicle reached ovulatory size (≥7 mm) was evaluated using ANOVA with a factorial design, considering the adjustments of the model and using the diameter of F1 on day 0 as a covariate. When the interaction was significant, the effect of treatment was evaluated on each follicular phase by orthogonal contrasts. The *p* value was set at .05.

3 | RESULTS

3.1 | Study 1

3.1.1 | Plasma P_4 concentrations and follicular activity during treatment with different intravaginal P_4 devices

Results are expressed as mean ± S.E.M. Plasma P_4 concentrations immediately before insertion (0 hr) averaged 0.35 ± 0.11 nmol/L in group 780 and 0.72 ± 0.32 nmol/L in group 160 ($p = .90$). Both intravaginal devices induced concentrations of P_4 higher than 3.2 nmol/L during the entire period of treatment. In Fig. 1, mean plasma P_4 concentrations during 8 days after insertion of the IVDs are shown. Plasma P_4 concentrations during treatment (day 0–7) averaged 19.6 ± 0.8 nmol/L in group 780 and 12.0 ± 1.0 nmol/L in group 160 ($p < .0001$). In group 780, plasma P_4 concentrations reached mean maximum values of 22.1 ± 2.0 nmol/L 1 hr after insertion. Afterwards, mean P_4 concentrations gradually declined reaching 14.4 ± 3.1 nmol/L at device withdrawal (day 7). In group 160, P_4 concentrations reached mean maximum concentrations of 17.6 ± 2.2 nmol/L 3 hr after insertion. Then, plasma P_4 concentrations declined reaching mean values of 6.2 ± 1.8 nmol/L at day 7. Finally, on day 8, P_4 concentrations were similar to that registered before insertion (0 hr) in both groups. Although both intravaginal devices induced luteal P_4 concentrations during the treatment period and the delivery profile was similar between devices, concentrations on group 780 were higher ($p < .05$) than in group 160 at all times, with the exception of sample of 3 hr in which the differences were not significant ($p = .26$). Moreover, the total amount of P_4 released during treatment was higher in group 780 than in group 160 registering AUC values of 145.9 ± 25.3 nmol $day^{-1}ml^{-1}$ and 72.0 ± 15.3 nmol day^{-1}/L, respectively ($p < .05$).

Figure 2 shows the mean maximum follicular diameter during treatment in both groups. Mean follicular diameter at insertion (day 0) in group 780 was 8.8 ± 1.0 mm and was similar to that of group 160 in which averaged 9.4 ± 1.2 mm ($p > .05$). Both intravaginal devices induced a decrease in maximum follicular diameter during treatment. On day 7, maximum follicular diameter in group 780 was 5.7 ± 0.3 mm and 6.9 ± 0.4 mm in group 160 ($p = .03$). On day 8, maximum follicular diameter in group 780 was 5.3 ± 0.3 mm and 7.2 ± 0.4 mm in group 160 ($P = .002$).

In females of group 780, the largest follicle present at insertion regressed and a new wave emerged on average 3.8 ± 1.0 days after insertion. Thus, the largest follicle (F2) present at withdrawal in females of this group always was originated from a new wave. Conversely, only

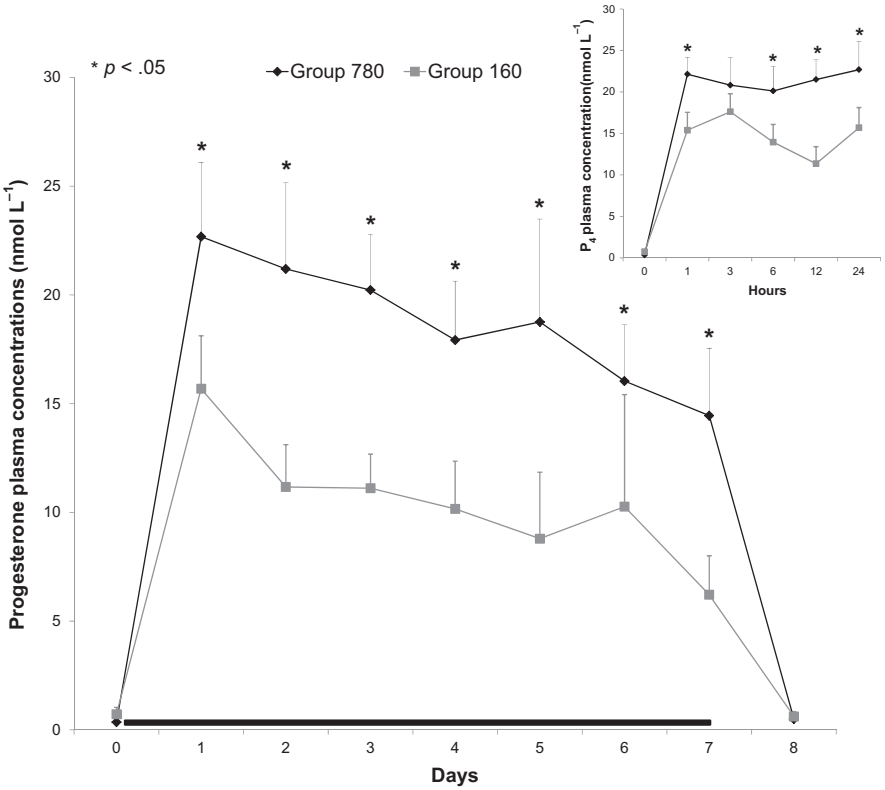

FIGURE 1 Mean plasma progesterone (P_4) concentrations immediately before insertion (day 0) until day 8 post-insertion of an intravaginal device containing 780 mg of P_4 (group 780; black diamond) or 160 mg of P_4 (group 160; grey square) in llamas ($n = 5$/group). The insert depicts the P_4 concentrations at 0, 1, 3, 6, 12 and 24 hr post-insertion. *Mean values differ between groups at $p < .05$. The black bar indicates the P_4 treatment period

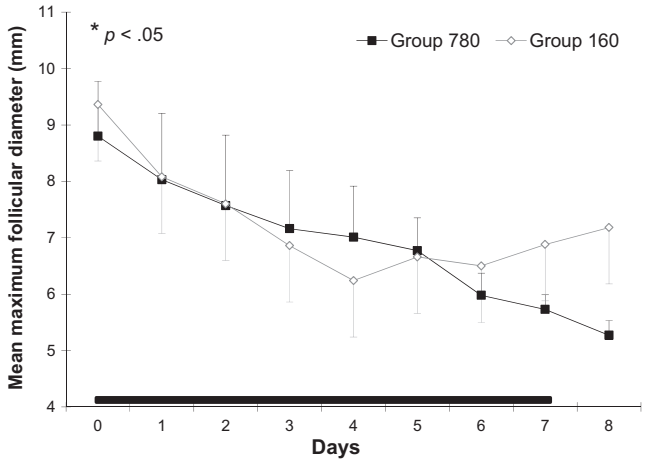

FIGURE 2 Mean maximum follicular diameter (mm) registered after insertion of an intravaginal device containing 780 mg of P_4 (group 780; black square) or 160 mg of P_4 (group 160; white diamond) in llamas (n = 5/group). *Mean values differ between groups (p < .05). The black bar indicates the P_4 treatment period

in 60% of the females of group 160, the treatment induced regression of the largest follicle present at insertion and a new wave emerged on average 1.0 ± 1.0 days after insertion. In the remaining 40% of the females of the group, the largest follicle present at insertion maintained a diameter of approximately 9 mm until device withdrawal and became a persistent follicle. Those follicles were detected for the first time 3 days before device insertion; thus, to determine the mean age of those follicles, the day of first detection was considered as day 0.

The largest follicle (F2) reached the ovulatory size at 10.4 ± 0.6 days in females of group 780 and at 8.0 ± 0.9 days after insertion in females of group 160 (p = .04). In addition, Fig. 3 shows the accumulative percentage of females with follicles ≥7 mm after device withdrawal in llamas of group 780 and 160. While 80 % (4/5 llamas) of females of group 160 had an ovulatory follicle 1 day after withdrawal, 80 % (4/5 llamas) of llamas of group 780 had an ovulatory follicle 4 days after withdrawal. The mean age of the largest follicle at device withdrawal averaged 3.2 ± 0.9 days in group 780 and 7.6 ± 1.1 days in group 160 (p = .02). In addition, the mean age when the largest follicle reached ovulatory size averaged 6.6 ± 0.3 days and 8.6 ± 0.7 days in group 780 and 160, respectively (p = .05). Furthermore, in llamas of group 160 that developed persistent follicles, the mean age at withdrawal averaged 10 days.

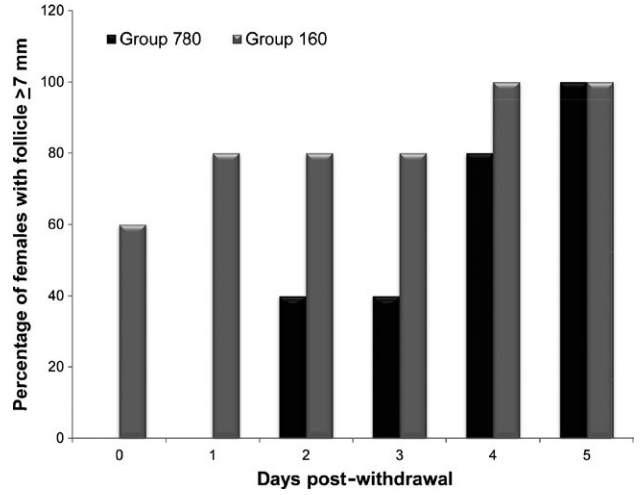

FIGURE 3 Accumulative percentage of females with largest follicle ≥7 mm after withdrawal of an intravaginal device containing 780 mg of P_4 (group 780; black bar) or 160 mg of P_4 (group 160; grey bar) in llamas (n = 5/group)

3.2 | Study 2

3.2.1 | Follicular activity in females, with follicles at different phases of development, treated with P_4 or no hormone

Results are expressed as mean ± S.E.M. Firstly, the effect of P_4 on follicular dynamics was evaluated considering the phase of development of the follicle on day 0 in animals treated with P_4 or none treated (control group). Follicular activity in treated and control llamas varied according to the phase of development of the follicle present on day 0 (Fig. 4):

Growing phase (Fig. 4a): Mean follicular diameter was smaller in treated than control group between days 2 and 13 of the study (p < .05). The beginning of follicular regression occurred on average at days 2.6 ± 0.6 and 10.3 ± 1.0 of the study in treated and control group, respectively (p < .0001). The emergence of F2 was registered at day 5.0 ± 0.2 in treated group and at day 9.8 ± 0.5 in the control group (p = .001). The mean age of the F2 at day 7 of the study (device withdrawal in the treated group) averaged 2.0 ± 0.2 days in the treated group and was first detected 2.8 ± 0.5 days later in the control group (p < .0001). In addition, the mean age of F2 when the follicle reached ovulatory size averaged 5.9 ± 0.6 days and 5.8 ± 0.5 days in the treated and control group, respectively (p = .83).

Plateau phase (Fig. 4b): Mean follicular diameter was smaller in the treated than the control group between days 6 and 12 of the study (p < .05). The beginning of follicular regression occurred on average at day 2.1 ± 0.6 and day 4.8 ± 1.3 in treated and control group, respectively (p = .06). The F2 emergence was registered on average at days 2.4 ± 0.8 and 3.3 ± 1.4 of the study in treated and control group, respectively (p = .5). The mean age of F2 at day 7 averaged 4.6 ± 0.8 days and 3.8 ± 1.4 days in the treated and control group, respectively (p = .6). Moreover, the age of F2 at ovulatory size averaged 6.6 ± 0.5 days in treated females and 5.3 ± 0.8 days in control females (p = .15).

Regression phase (Fig. 4c): Mean follicular diameter did not differ between treated and control group (p > .05). The F2 emergence differ between treated and control group occurring at day 4.1 ± 0.8 of the study and 2.3 ± 1.6 days before the beginning of the study, respectively (p < .0001). In the treated group, the effect of IVD insertion on F2 emergence originated two different situations: in three females, treatment had no effect on F2 emergence that was registered on average at 2.0 ± 0.6 days after insertion (day 0) and the growth of F2 continued until device withdrawal on day 7 of the study. Conversely, in the

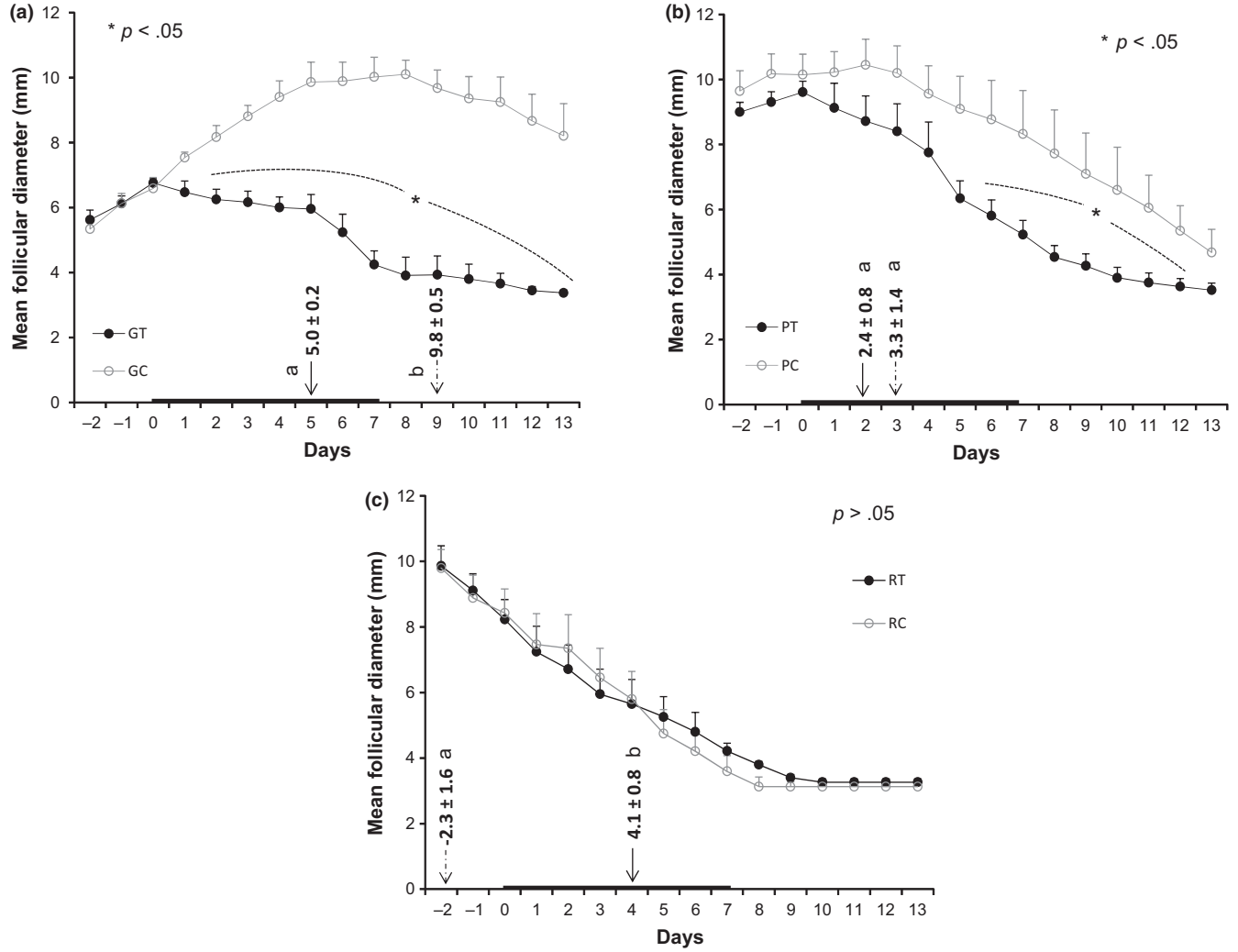

FIGURE 4 Mean diameter (mm) of the largest follicle (F1) at the growing (**a**), plateau (**b**) or regression (**c**) phase at beginning of the study (day 0) in females treated with an intravaginal device containing 780 mg of P_4 (closed circle) or in none-treated animals (open circle). The arrows show the day of emergence of the largest follicle (F2) of a new wave in treated (continuous arrow) and control females (discontinuous arrow). *Mean values differ between groups at $p < .05$. Different letters show differences between means (a vs b $p < .05$). The black bar indicates the P_4 treatment period in treated females. GT, Growing treated; PT, Plateau treated; RT, Regression treated; GC, Growing control; PC, Plateau control; RC, Regression control

remaining females ($n = 4$), F2 emerged on average at 6.0 ± 0.4 days after insertion. On day 0 (device insertion), these females had a follicle at the regression phase and a second follicle at the growing phase that emerged 2.8 ± 0.5 days before insertion and had a mean diameter at day 0 of 5.2 ± 0.2 mm. The development of the second growing follicle was suppressed by treatment inducing the emergence of a new follicle (F2). The mean F2 diameter at device withdrawal (day 7) in the three first females was 5.9 ± 0.2 mm, while, in the four remaining females averaged 4.2 ± 0.4 mm ($p = .02$). The mean age of F2 at day 7 of the study was 2.7 ± 0.9 days and 9.3 ± 1.5 days in the treated and control group, respectively ($p = .003$). Additionally, the mean age of F2 at ovulatory diameter was 6.3 ± 0.5 days in treated females and 6.5 ± 0.3 days ($p = .76$). Within the treated group in the three females in which F2 was emerging at device insertion, the mean age of F2 at day 7 (device withdrawal) was 5.0 ± 0.6 days and 7.3 ± 0.7 days at ovulatory diameter. Conversely, in four females that had on day 0 of the study, besides the regressive follicle, a second growing follicle, the F2 had a mean age at device withdrawal of 1.0 ± 0.4 days ($p = .002$) and 5.5 ± 0.3 days at ovulatory diameter ($p = .038$).

Finally, follicular activity was evaluated in treated and control females independently of the phase of development of the follicle on day 0. The IVD 780 promoted follicular regression and induced emergence and development of a new follicle (follicular turnover) in all females regardless of the phase of the follicular wave on day 0 of the study. Mean follicular diameter at day 0 was 8.4 ± 0.5 mm in control group and 8.2 ± 0.4 mm in treated group ($p > .05$). The beginning of follicular regression occurred on average at day 1.9 ± 0.3 and day 5.3 ± 0.9 of the study in treated and control group, respectively ($p = .002$). Mean follicular diameter was significantly smaller in the treated group between days 3 and 13 of the study ($p > .05$, Fig. 5).

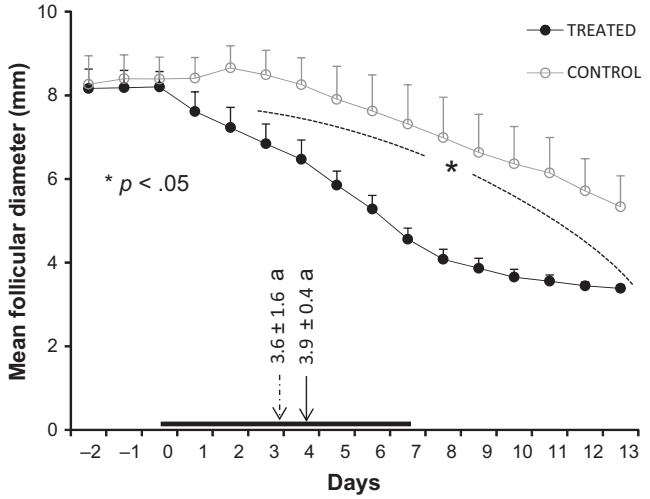

FIGURE 5 Mean diameter of the largest follicle present at insertion (F1), in females treated with P_4 (closed circle) or in none-treated animals (open circle) regardless of the phase of the follicular wave at insertion (day 0). The arrows show mean day of emergence of the largest follicle of a new wave (F2) in control (discontinuous arrow) vs treated group (continuous arrow). *Mean values differ between groups at $p < .05$. Similar letters show no differences between means ($^a p > .05$). The black bar indicates the P_4 treatment period in treated females

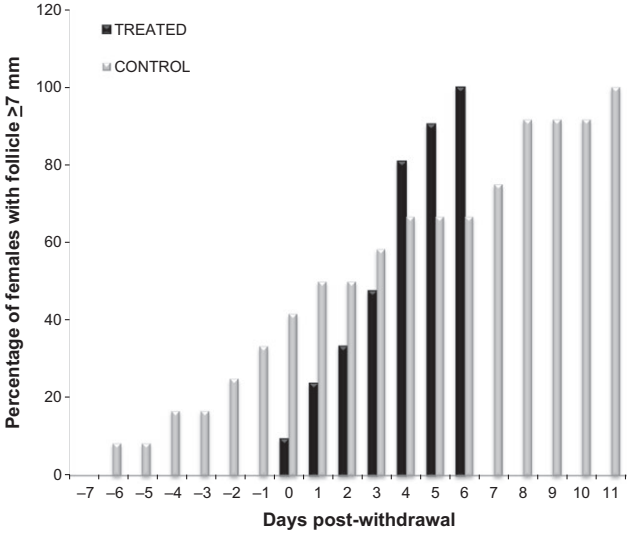

FIGURE 6 Accumulative percentage of females with largest follicle from a new wave (F2) ≥7 mm in animals treated with an intravaginal device (IVD) containing 780 mg of P_4 (black bar) or in none-treated females (grey bar). Day 0: IVD withdrawal in treated females

Mean emergence of F2 was registered at day 3.9 ± 0.4 and day 3.6 ± 1.6 of the study in the treated and control group, respectively ($p = .5$). The F2 emergence occurred from one day before the beginning of the study (day 0) until day 7 of the study in the treated group and between day 5 until day 11 of the study in the control group. The mean age of F2 at day 7 of the study (device withdrawal in treated animals) was 3.1 ± 0.5 days in the treated group and 3.4 ± 1.6 days in the control group ($p = .8$). In the treated group, F2 reached the ovulatory size between day 7 and 13 of the study (day 0 and 6 after device withdrawal) while in control females occurred between day 1 until day 18 of the study ($p < .05$; Fig. 6). The mean age of F2 at ovulatory size was 6.2 ± 0.2 days in the treated group and 5. 8 ± 0.3 days in the control group ($p = .32$).

3.2.2 | Hormonal pattern after treatment

In treated animals, E_2 production increased with F2 development from IVD withdrawal until the follicle reached its ovulatory size. When F2 reached ovulatory size, plasma E_2 concentrations were not different between the treated and control group ($p > .05$). Finally, in all females, the injection of 16.8 μg of buserelin induced ovulation of F2 resulting in the disappearance of the dominant follicle by 48 hr after injection. In 11 treated females, plasma P_4 concentrations by days 4 and 8 after buserelin injection averaged 3.2 ± 1.2 and 14.6 ± 1.5 nmol/L, respectively.

4 | DISCUSSION

To our knowledge, this is the first study comparing plasma P_4 concentrations and their effect on follicular activity in llamas treated with IVDs containing different P_4 doses (160 or 780 mg). In addition, the effect of P_4 on follicular activity in llamas with follicles at all stages of development at the beginning of the treatment with P_4 was also evaluated and compared with a control group without treatment. Plasma P_4 concentrations in females before IVDs insertion (0 hr) were below 1.9 nmol/L indicating the absence of a functional CL at the start of the treatment as its presence is associated with plasma P_4 concentrations above 3.2 nmol/L (Aba, 1995; Bravo et al., 1991).

As previously reported in cows (Kojima et al., 1992; Macmillan, Taufa, Barnes, & Day, 1991; Roberson, Wolfe, Stumpf, Kittok, & Kinder, 1989; van Werven, Waldeck, Souza, Floch, & Englebienne, 2013) and ewes (Scudamore, McEvoy, Aitken, Robinson, & Robertson, 1993), although both intravaginal devices induced luteal concentrations of P_4, the treatment with higher P_4 content was related with greater plasma P_4 concentrations. In addition, peak concentrations induced by the intravaginal device containing 780 mg of P_4 in the present study were lower than those registered immediately after insertion of the IVD CIDR® containing 330 mg of P_4 in llamas (Chaves et al., 2002). However, after CIDR® insertion, concentrations sharply decreased until day 3 after insertion, while the device containing 780 mg in the present study maintain concentrations around maximum until device withdrawal. Besides different P_4 contents in the devices, it has been suggested in llamas (Aller et al., 2015) and cows (van Werven et al., 2013) that differences in contact surface area between devices result in different P_4 plasma concentrations patterns. It could be speculated that in the present study, differences between devices hormone release may partly be responsible for the observed patterns.

The dose of 780 mg was more efficient to induce the reduction of the maximum follicular diameter than the 160 mg dose. Thus, treatment with the highest content promoted follicular regression and the emergence of a new wave and one follicle was selected and reached

ovulatory diameter on average 5 days after device withdrawal. These findings are in agreement with previous reports in llamas treated with intravaginal sponges containing medroxyprogesterone acetate (MAP) for 8 days (Aller et al., 2010) or injectable P_4 (Santiani et al., 2002). The latter authors have reported that P_4 injection in llamas induced regression of the dominant follicle and the emergence of a new wave after progesterone effect had ceased. Moreover, Aller et al. (2010) observed that the application of an ovulatory stimulus 12 days after sponge insertion induced ovulation in all llamas demonstrating the existence of a mature follicle at this time. In cows and ewes, only P_4 treatments that induce luteal concentrations of the hormone in plasma effectively suppress LH pulsatility inducing regression of the dominant follicle, hastening the emergence of a new wave and ensuring the presence of a mature and healthy follicle at the end of treatment allowing oestrous synchronization without comprising fertility (Bergfeld et al., 1996; Kojima et al., 1992; Leyva et al., 1998; Menchaca & Rubianes, 2004; Sirois & Fortune, 1990). The findings of the present study suggest that plasma P_4 concentrations induced by the intravaginal device containing 780 mg of P_4 were effective to induce follicular regression and the emergence of a new wave (follicular turnover) resulting in the development of a young follicle in a definite time after treatment.

The observation that in the females treated with the intravaginal device containing 160 mg of P_4, follicles with ovulatory diameter were present earlier than in those females treated with the device containing 780 mg of P_4 was in relation with the persistence of the follicle present at insertion in some females treated with the dose of 160 mg. These results are in agreement with those registered in cows and ewes in which subtle changes in P_4 concentrations could result in radical alterations of follicular dynamics by inducing different patterns in LH pulsatility (Rubianes & Menchaca, 2003; Seekallu et al., 2009; Sirois & Fortune, 1990). Thus, in the treatments with synthetic progestagens (Kojima et al., 1992; Viñoles, Forsberg, Banchero, & Rubianes, 2001) or when P_4 concentrations induced by treatment are subluteal (Johnson, Dailey, Inskeep, & Lewis, 1996; Kojima et al., 1992), increased LH pulsatility is observed resulting in the persistent growth of the dominant follicle that produced higher E_2 levels leading to the ovulation of aged follicles and compromising fertility (Adams et al., 1992b; Austin et al., 1999; Bergfeld et al., 1996; Seekallu et al., 2009; Sirois & Fortune, 1990; Viñoles et al., 1999). In cows, it has been reported that a follicular dominance period greater than 9 days affects negatively pregnancy rates (Austin et al., 1999). Based on the previous observations, it could be suggested that P_4 concentrations induced by the intravaginal device containing 160 mg of P_4 failed to induce follicular turnover in all females leading to the development of aged follicles. Therefore, the intravaginal device containing 780 mg would be more appropriate to control follicular activity in llamas. Hence, based on results of *Study 1*, the intravaginal device containing 780 mg was selected for *Study 2*, in order to evaluate whether follicular diameter at the beginning of P_4 treatment could affect its effectiveness to control follicular activity.

The intravaginal device containing 780 mg of P_4 suppressed follicular activity regardless of the stage of the follicle present at insertion and induced the emergence of a new wave around day 4 after insertion. Thus, follicular regression started earlier in P_4-treated than untreated females. These observations are similar to that previously reported in llamas treated with intravaginal sponges containing MAP (Aba et al., 1999; Aller et al., 2010) or the IVD CIDR® containing 330 mg of P_4 (Chaves et al., 2002). Furthermore, the administration of injectable P_4 for 2 or 4 (Santiani et al., 2002), 5 (Carretero et al., 2006) or 7 days (Alberio & Aller, 1996) induced follicular regression in llamas. In addition, Aller et al. (2010) reported that progestagen treatment in llamas results in the emergence of a new wave around 4 days after insertion. Conversely, other authors (Ferrer, Agüero, Flores, & Rutter, 1999) observed that the treatment with intravaginal sponges containing MAP is not effective to control follicular activity in llamas.

In the present study, P_4 treatment reduced the dispersion of F2 emergence. Yet, the moment of F2 emergence depended on the follicular phase at insertion. In cows and ewes, P_4 affects LH concentrations without a direct effect on systemic concentrations of FSH. Yet, through the suppression of the dominant follicle, P_4 hasten the FSH surge that preceeds new wave emergence (Adams et al., 1992a,b; Baby & Bartlewski, 2011; Seekallu et al., 2009). It could be suggested that a similar situation would occur in llamas. All females treated with the intravaginal device containing 780 mg of P_4 had a growing follicle originated from a new wave with a mean age of approximately 3 days (range 2–5 days) at device withdrawal. This observation is in agreement with that reported in goats treated with a short P_4 protocol (Menchaca & Rubianes, 2004; Rubianes & Menchaca, 2003). Thus, the evaluated treatment resulted in the development of a new follicle after device withdrawal in all females.

The evaluated P_4 treatment resulted effective to concentrate the appearance of new follicles with ovulatory diameter approximately day 6 after device withdrawal, reducing in 11 days the range of appearance of those follicles, respect to animals that remain untreated (control group). In addition, plasma E_2 concentrations were positively related to F2 development after withdrawal and E_2 production by F2, when they reached 7 mm, was similar between treated and control females. In addition, P_4 concentrations on day 4 and 8 after GnRH injection in females treated with the intravaginal device with 780 mg of P_4 resemble those registered after ovulation in llamas mated in the presence of an ovulatory follicle (Aba, 1995). These observations allow suggesting that in terms of E_2 production and ovulatory capacity, F2 was similarly functional in treated and control animals. Thus, the selected device would be used without the need to check the ovaries status before initiation of treatment, which would be extremely impractical. Therefore, the protocol hereby evaluated results effective to synchronize follicular activity regardless of the follicular diameter at start of treatment. Whether the addition of other hormones to the protocol might reduce even more the range of appearance of ovulatory follicles after treatment remains to be elucidated.

ACKNOWLEDGEMENTS

This research was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT N° 2074). M.V. Cavilla and C.P. Bianchi are holders of fellowships from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

CONFLICTS OF INTEREST

None of the authors have any conflict of interests to declare.

AUTHOR CONTRIBUTIONS

Cavilla María Verónica has participated in developing the design of the experiment. She has carried out the experiment, collected and analysed the data and drafted the manuscript. Bianchi, Carolina Paula, Aguilera Florencia and Hermida María have contributed in carrying out the experiment and with contents of the manuscript. Aba Marcelo Alfredo as the director of the doctoral project of Miss Cavilla María Verónica has participated in the design of the study and has contributed in carrying out the experiment, with the analysis of the data and revising critically the contents of the manuscript. All authors have read and approved the final manuscript.

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