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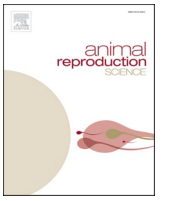

# Intravaginal progesterone device reinsertion during the early luteal phase affects luteal function and embryo yield in superovulated ewes

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ABSTRACT

This study checked the efficacy of progesterone (P4) device reinsertion during the early luteal phase on luteal function and embryo yield in superovulated crossbred ewes. Twenty multiparous ewes received an intravaginal P4 device for nine days (D0 to D9) followed by six decreasing doses (25, 25, 15, 15, 10, 10%) of 133 mg pFSH i.m. at 12 h intervals, starting 60 h before P4 device removal. Ewes were naturally mated at 12 h intervals while in estrus. On D13, ewes with viable corpora lutea (CL; n = 19) were equally allocated for receiving their P4 device reinsertion (G-P4; n = 10) or not (G-Control; n = 9). On D17, the P4 device was removed, and all females received the cervical relaxation protocol 16 h to 20 min before non-surgical embryo recovery. CL count and their functionality classification were performed on D13 and D17 by transrectal B-mode and color Doppler ultrasonography (US). Plasma P4 concentrations (ng/mL) of G-P4 ewes increased ($P < 0.05$) over the days, being greater on D17 (9.2 ± 2.8) than on D9 (1.9 ± 0.7) and D13 (1.6 ± 0.4). The overall CL count per ewe tended to be greater ($P = 0.09$) in G-P4 compared with G-Control. The occurrence of premature regression of corpora lutea did not differ ($P > 0.05$) between G-P4 (30.0%) and G-Control (44.4%). The number of ova/embryos recovered was greater ($P < 0.05$) in G-P4 (11.6 ± 2.9) compared with G-Control (3.7 ± 2.0), respectively. Altogether, the reinsertion of the P4 device for four days after superovulation in ewes promotes greater P4 concentrations, resulting in greater ova/embryos recovered.

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## 1. Introduction

Multiple ovulation and embryo transfer (MOET) programs remain the most prominent source for supporting embryo production in sheep (Souza-Fabjan et al., 2021). In addition to many intrinsic and extrinsic factors negatively affecting embryo yield (Bartlewski et al., 2016) up to ovulation, corpora lutea (CL) generated are associated with the maintenance of the adequate uterine environment needed for early embryo development. When superovulated, small ruminants are more likely to undergo premature regression of corpora lutea (PRCL), reaching up to 58% of females (Saharrea et al., 1998; Oliveira et al., 2018). The exogenous gonadotropins used for superovulation might be one of the causes of PRCL in superovulated ewes; and, in fact, a lower total dose of porcine follicle-stimulating hormone (pFSH; 100 mg) was related to a greater percentage of donor ewes with normal CL function compared with higher doses (133 and 200 mg) (Bevilaqua et al., 2023). Interestingly, PRCL was not associated with the season, donor age, body condition score, or the number of MOET repetitions (Rocha et al., 2022). The occurrence of PRCL determines embryo quality, since it results in low progesterone (P4) concentrations (Cervantes et al., 2007), directly affecting the uterine milieu and embryo development (Forde and Lonergan, 2017).

In small ruminants, strategies tested to reduce PRCL occurrence are focused either on maintaining CL function by administering human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GnRH) during the early luteal phase (Saharrea et al., 1998; Dias et al., 2022) or inhibiting PGF2-α synthesis, by using flunixin-meglumine (Figueira et al., 2020; Fonseca et al., 2022). In a recent study, an attempt to avoid potential detrimental effects of PRCL on embryos by administering hCG four days after the last FSH dose was associated with increased P4 concentration on the day before non-surgical embryo recovery (NSER), followed by greater embryo recovery rate compared with the control group (Dias et al., 2022). Another less explored possibility is the supplementation of P4 during the early luteal phase. In this way, the use of intravaginal sponges impregnated with fluorogestone acetate immediately after the last matting improves embryo yield in superovulated goats, although the PRCL occurrence was increased (s et al., 2007).

It was previously demonstrated that a potential P4 content remained in intravaginal silicon devices impregnated with P4 previously used for 10 days (Pinna et al., 2012). In this study, similar plasma P4 concentrations were observed until five days after the P4 device insertion among ewes with new, once-used (five days), and twice-used P4 devices (10 days). Thus, we hypothesized that the reinsertion during the early luteal phase of a previously used P4 device could increase the P4 plasma concentrations, and consequently, mitigate the PRCL negative effects on luteal function, increasing embryo yield in superovulated ewes.

## 2. Materials and methods

### 2.1. Ethics, location, and experimental animals

This research was approved by the Animal Care Committee of Universidade Federal Fluminense, Brazil (protocol 3614141221). The experiment was performed in September, during the local non-breeding season (Balaro et al., 2014). Twenty clinically healthy

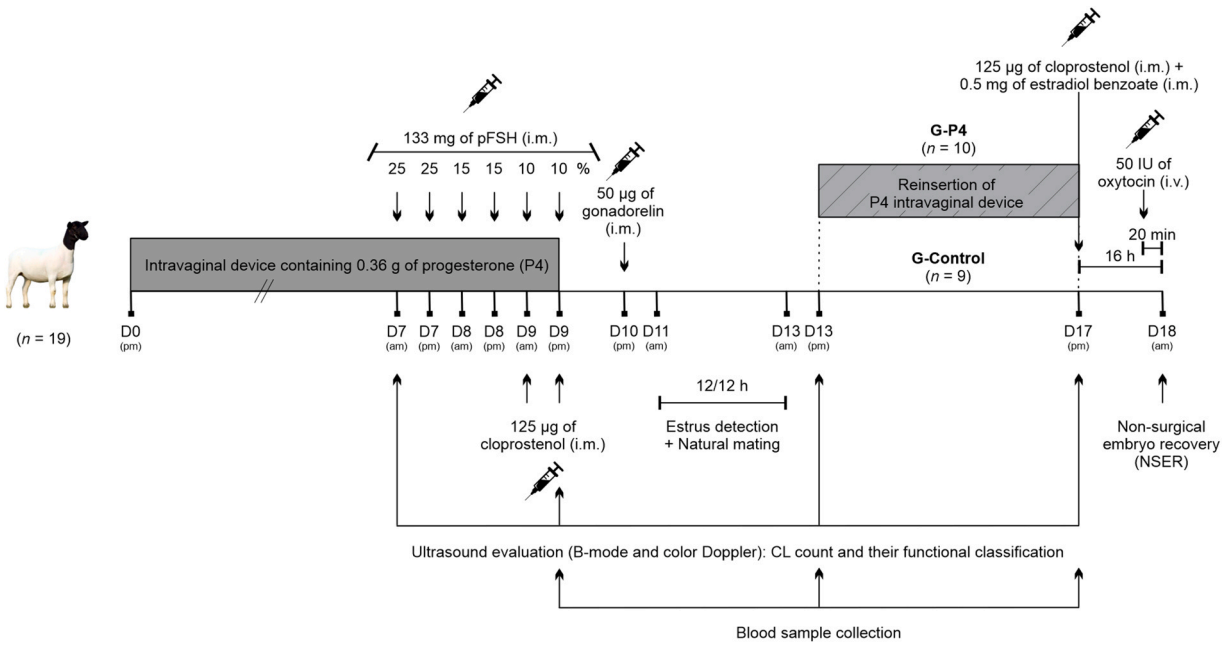


**Fig. 1.** Schematic representation of the experimental procedures designed to evaluate the effects of reinsertion of progesterone (P4) device (G-P4 vs. G-Control) in superovulated crossbred Dorper/Santa Inês ewes that underwent non-surgical embryo recovery (NSER). pFSH: porcine follicle stimulating hormone; Folltropin-V®; i.m. intramuscular; i.v. intravenous; CL: corpora lutea.

crossbreed Dorper/Santa Inês sheep, housed in a commercial farm in Aracitaba, MG, Brazil (latitude 21°19′ S, longitude 43°19′W, altitude 657 m) were used. Animals were kept in collective pens and received a diet of two meals with *Pennisetum purpureum schum* or corn silage supplied to the trough, and mineral salt and water ad libitum. The mean body condition score (BCS: 1 =very thin and 5 =very fat) was 3.2 ± 0.1, and the mean ( ± standard deviation) body weight of ewes at the beginning of the study for G-Control and G-P4 was 59.7 ± 11.8 kg and 62.7 ± 7.8, respectively.

### 2.2. Treatments and experimental groups

There is a simplified schematic depiction of experimental procedures in Fig. 1. For synchronous estrus induction, all ewes received an intravaginal silicone device containing 0.36 g of P4 (Primer PR®; Tecnopec, Embu-Guaçu, Brazil) that was inserted on a random day of the anovulatory period (D0) and maintained for 9 days (D0 – D9). A total of 133 mg pFSH (Folltropin-V®, Vetoquinol, Mairiporã, Brazil) was intramuscularly administered in six decreasing doses (25–25–15–15–10–10%) at 12 h intervals (06:00 AM and 06:00 PM) starting 60 h before P4 device removal. All ewes also received two i.m. injections of 125 μg of cloprostenol (Sincrocio®; Ouro Fino, Cravinhos, Brazil) at the fifth and sixth pFSH doses, and 50 μg of gonadorelin i.m. (Gestran®, Tecnopec, São Paulo, Brazil) at 24 h after P4 device removal. Ewes were naturally mated for fertile rams up to three times at 12 h intervals while in estrus. All previously used P4 devices were washed with running water, dried at room temperature, inserted into identified bags, and kept at 4 ºC until reinsertion in the same female. On D13, mated ewes were allocated into two experimental groups according to their body weight and body condition score for either receiving the P4 device reinsertion (G-P4; $n = 10$) or not (G-Control; $n = 9$). In G-P4, the reinserted P4 device was removed on D17.

### 2.3. Ovarian ultrasonographic evaluation

Ultrasonographic evaluations (B- and color Doppler mode) were performed in all ewes at the time of the P4 device reinsertion (D14) and at its removal (D17; 16 h before NSER to determine the CL count and their functional classification). Ovaries were scanned with a portable ultrasound scanner (M5 Vet®; Mindray, Shenzen, China) equipped with an 8.0 MHz transrectal transducer taped to a stiffening PVC tube to facilitate external manipulation. Ewes were examined in a standing position, fecal pellets were removed manually from the rectum, and 10 mL of carboxymethylcellulose gel (Carbogel UTL; Carbogel Indústria e Comércio Ltda, São Paulo, Brazil) was deposited with a syringe into the rectum.

Each CL was carefully scanned by color Doppler-mode ultrasound to detect color signals indicative of tissue blood perfusion, and consequently, their functionality classification, as normal or prematurely regressed (PRCL, corpora lutea in which no signs of blood perfusion were identified). The definition of this criterion was based on the ultrasonographic characteristics of luteal tissue in a period of luteolysis in ewes (Figueira et al., 2015). The Color Doppler settings were maintained during all evaluations [Pulse repetition frequency (PRF): 1.0 kHz; Gain: 72%; Depth: 4.3 cm; CFM (Frequency): 4.6 MHz; and Wall Filter (WF): 1].

### 2.4. Plasma progesterone concentrations

Blood was sampled via the jugular vein into vacutainer tubes containing EDTA on D9 (immediately before the P4 device removal from estrus induction protocol), D13 (immediately before P4 device reinsertion), and D17 (immediately before reinserted P4 device removal). Blood samples were centrifuged at 1000 *g* for 15 min, plasma was recovered and stored at − 20 °C until the P4 dosage assay was performed. Plasma P4 values were determined using a solid-phase radioimmunoassay utilizing a commercial kit (MP Diagnostics Division, Orangeburg, New York, USA). Samples were analyzed in a single assay, with a sensitivity of 0.05 ng/mL and an intra-assay coefficient of variation of 9.1%, with all values within the curve.

### 2.5. Non-surgical embryo recovery procedures

The NSER was performed on D18 (8.5 days after the first device removal), after the cervical dilation protocol, which started with 125 μg of cloprostenol (Sincrocio®; Ouro Fino, Cravinhos, Brazil) and 0.5 mg of estradiol benzoate (Gonadiol®; Zoetis, Campinas, Brazil) i.m. 16 h (D17) and 50 IU of oxytocin (Ocitocina Forte®, UCB, Jaboticabal, Brazil) 20 min before NSER. The anesthetic procedure consisted of the administration of 10 mL of a hyoscine/dipyrone solution being a half i.v. and another half i.m. (Buscofin Composto®; Agener União, Taboão da Serra, Brazil), 2 mL of lidocaine/xylazine solution (Bloc®; JA Animal, Patrocinio Paulista, Brazil) for epidural block, 5 mL of lidocaine 2% (Lidovet®; Bravet Ltda, Rio de Janeiro, Brazil) soaked in latero-lateral cotton to the opening of the caudal ostium of the cervix. The NSER was performed according to the previously described by Figueira et al. (2020). Ova/zona pelucidae/embryos recovered were morphologically evaluated using a stereomicroscope with a magnification of 40X, while using the same criteria as those for cattle (Mapletoft et al., 2020); the classification considers the stage of development of the embryo according to its lifetime and the number of cells extruded, among others. Grade 1, 2, and 3 embryos were considered viable embryos. Grade 1 and 2 embryos were cryopreserved and stored (Fonseca et al., 2018).

### 2.6. Variables and statistical analysis

The following endpoints were recorded: estrus response (%); ewes with at least one CL on the day before NSER (%); overall CL count per ewe; ewes with at least one PRCL (%); number of PRCL per ewe; overall PRCL rate per ewe (number of PRCL x 100 / overall

number of CL); ewes successfully penetrated and flushed (%); overall CL count per ewe flushed; number of ova/zona pelucidae/embryos recovered; recovery rate (%); number of viable embryos recovered, unfertilized ova and zona pelucidae; embryo viability rate (%); and ewes with ova/zona pelucidae/embryos and viable embryos recovered (%).

Statistical analysis was performed using the software BioEstat 5.3, Belém, Brazil, and IBM SPSS® Statistics, version 19. Non-parametric data were analyzed by the Mann-Whitney test (Ova/zona pelucidae/embryos recovered, Recovery rate, Viable embryos recovered, Unfertilized ova and Zona pelucidae), parametric data were analyzed by t-test (Overall CL count per ewe, Number of PRCL and Overall CL count per ewe flushed) and ANOVA (Plasma P4 concentration (dependent) vs. days (independent)), also for repeated measures followed by posthoc LSD. The homogeneity of variances was checked by the Levene test, when variances were not homologous, data were Log 10 transformed for ANOVA. Frequencies were assessed by Chi-square (Overall PRCL rate) or Fisher's exact test (Estrus response, Ewes with at least one CL on the day before NSER, Ewes successfully penetrated and flushed, Embryo viability rate, Ewes with ova/zona pelucidae/embryos recovered and Ewes with viable embryos recovered). The results are shown as means ± standard error of means (MEANS ± SEM). Differences were considered significant when $P < 0.05$, or as otherwise noted.

## 3. Results

Plasma P4 concentrations of G-P4 ewes increased ($P < 0.05$) over the days and tended to be greater ($P = 0.055$) on D17 compared with G-Control ewes (Fig. 2). Data related to estrus and ovarian responses, as well as NSER are presented in Table 1. The estrus response and percentages of ewes with at least one CL on the day before NSER did not differ ($P > 0.05$) between groups. The overall CL count per ewe tended to be greater ($P = 0.09$) in G-P4 compared with G-Control. The percentage of ewes with at least one PRCL on the day before NSER, the number of PRCL per ewe, and the overall PRCL rate per ewe were similar ($P > 0.05$) in both groups.

The percentage of ewes successfully penetrated and flushed was superior that 66% in both groups. From ewes in which NSER was unsuccessful ($n = 6$), cervical transposing was possible only with a Hegar dilator in three ewes (G-Control = 1 and G-P4 = 2). In two (G-Control = 1 and G-P4 = 1), cervical os could not be clipped because of vestibulo-vaginal stenosis, and cervical transposing was incomplete by Hegar dilator in the other one (G-Control=1).

The overall CL count per ewe flushed did not differ ($P > 0.05$) between groups. In G-P4 the number of ova/zona pelucidae/embryos recovered was greater ($P < 0.05$) compared with G-Control. The recovery rate tended to be greater ($P = 0.054$) in G-P4 ewes compared with the control ones. The other variables of embryo yields were similar ($P > 0.05$) in both groups.

## 4. Discussion

To the best of our knowledge, this is the first study aiming to supplement P4 after estrus/ovulation (i.e., during the early luteal phase) through the reinsertion of previously used P4 devices in superovulated ewes. The postulated hypothesis that the own reinserted P4 device could significantly enhance P4 concentrations and mitigate potential detrimental effects of PRCL enough to positively affects the NSER-associated parameters was confirmed. This study provides, therefore, an important field-applied strategy for circumventing possible harmful effects of PRCL in superovulated donor ewes.

The P4 device reinsertion strategy tested in the present study was not able to fully prevent the occurrence of PRCL (30.0% for G-P4 vs. 44.4% for the G-Control group; $P > 0.05$), however, the time of reinsertion did not increase the PRCL rate as observed in donor

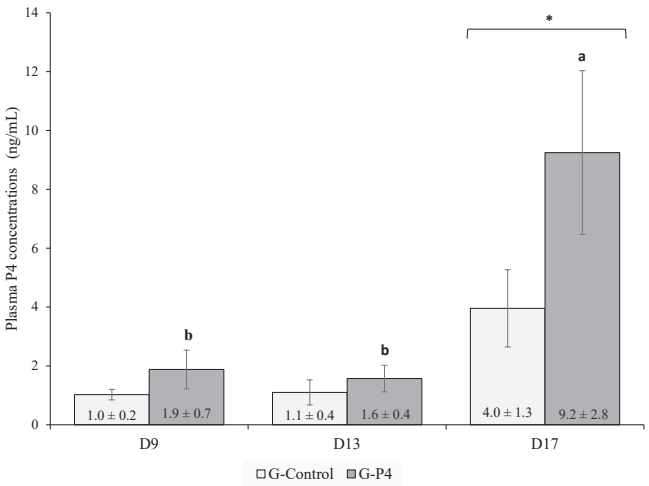


**Fig. 2.** Plasma progesterone (P4) concentration of superovulated ewes immediately before the P4 device removal from the estrus induction protocol (D9), at the P4 device reinsertion (D13), and at immediately before the reinserted P4 device removal (D17; 16 h before non-surgical embryo recovery procedure) in crossbred Dorper/Santa Inês ewes submitted to a 9-day progesterone-based estrus induction protocol associated with a superovulatory treatment using six decreasing doses of pFSH (133 mg, Folltropin-V®, from 60 h before P4 device removal). [a,b] Means with different superscripts between days within the G-P4 group ($P < 0.05$). * $P = 0.055$ for statistical comparison between groups on D17.

**Table 1**
Endpoints (% or mean ± S.E.M.) of crossbred Dorper/Santa Inês ewes receiving (G-P4) or not (G-Control) reinsertion of intravaginal progesterone (P4) device, for four days, during the early luteal phase promoted by synchronous estrus induction protocol followed to superovulatory treatment* .

| Endpoints | G-Control | G-P4 | *P value* |
|---|---|---|---|
| Estrus response (%) | 90.0 (9/10) | 100.0 (10/10) | n.s. |
| Ewes with at least one CL on the day before NSER (%) | 100.0 (9/9) | 100.0 (10/10) | n.s. |
| Overall CL count per ewe | 9.8 ± 2.4 [88] | 18.3 ± 4.4 [183] | 0.09 |
| Ewes with at least one PRCL on the day before NSER (%) | 44.4 (4/9) | 30.0 (3/10) | n.s |
| Number of PRCL per ewe | 2.8 ± 1.9 [25] | 1.4 ± 0.7 [14] | n.s |
| Overall PRCL rate per ewe (%) | 35.7 ± 15.0 | 14.6 ± 7.5 | n.s |
| Ewes successfully penetrated and flushed (%) | 66.7 (6/9) | 70.0 (7/10) | n.s. |
| Overall CL count per ewe flushed | 12.7 ± 2.8 [84] | 23.0 ± 5.3 [177] | n.s. |
| Ova/zona pelucidae/embryos recovered | 3.7 ± 2.0 [22] | 11.6 ± 2.9 [81] | 0.03 |
| Recovery rate (%) | 24.5 ± 10.6 [22] | 57.3 ± 10.5 [81] | 0.054 |
| Viable embryos recovered* * | 0.3 ± 0.3 [2] | 2.7 ± 1.8 [19] | n.s. |
| Unfertilized ova | 2.3 ± 2.1 [17] | 8.0 ± 2.7 [57] | n.s. |
| Zona pelucidae | 0.5 ± 0.3 [3] | 0.7 ± 0.7 [5] | n.s. |
| Embryo viability rate (%) | 9.1 (2/22) | 23.5 (19/81) | n.s. |
| Ewes with ova/zona pelucidae/embryos recovered (%) | 66.7 (4/6) | 100.0 (7/7) | n.s. |
| Ewes with viable embryos recovered (%) | 16.7 (1/6) | 57.1 (4/7) | n.s. |

() proportion and [] total of number of variables involved.
* Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing doses of pFSH (133 mg, Folltropin-V®, from 60 h before P4 device removal) and two intramuscularly (i.m.) injections of 125 μg of cloprostenol at the fifth and sixth pFSH doses, and 50 μg of gonadorelin i.m. at 24 h after P4 device removal.
* *Grade 1, 2, and 3 embryos.

goats receiving fluorogestone-impregnated sponges after the last mating (Cervantes et al., 2007). Interestingly, these authors counted both types of CL (normal or in regression) by visual classification during laparotomy, while in the present study, a similar count was performed but taking into account ultrasonography features as proposed earlier (Oliveira et al., 2018; Rodriguez et al., 2019; Bevilaqua et al., 2023).

Previous results in superovulated Santa Inês ewes (Rodriguez et al., 2019) and Dorper ewes (Rocha et al., 2022), that were not subject to any anti-luteolytic strategies, reported experimental groups with up to 45% and 25% of CLs with morphological characteristics of premature regression, respectively. Although the cascade causing PRCL was possibly already in progress at the time of reinsertion of the P4 device, the strategy tested in the present study indicates that additional PRCL was avoided. The PRCL is a major cause of naturally occurring subfertility in ruminants (Yaniz et al., 2008) and its occurrence is exacerbated in superovulated animals (Okada et al., 2000; Oliveira et al., 2018; Rodriguez et al., 2019; Rocha et al., 2022; Bevilaqua et al., 2023), and can occur in up to 75% of females (Fukui et al., 1998). Although multifactorial causes have been posited, this phenomenon is not completely elucidated. There are indications of being initially triggered by changes in hormonal profiles (Cognie et al., 1982; Braden et al., 1989; Lamming and Mann, 1995; Saharrea et al., 1998; Chemineau et al., 2006), and/or in follicular (Cognie et al., 1982; Stubbings and Walton, 1986; Chemineau et al., 2006) and luteal cells (Braden et al., 1989; Okada et al., 2000; Chemineau et al., 2006).

P4 is synthesized by luteal cells and plays a crucial autocrine role in cellular function. Due to the insufficient concentrations of P4, the chain responsible for the release of oxytocin and prostaglandin is more sensitive to estrogens (Chemineau et al., 2006), consequently, CLs are more sensitive to luteolytic substances (Braden et al., 1989). In addition, P4 is known to downregulate the expression of oxytocin receptors in the luminal epithelium (Wathes et al., 1996). Despite not being investigated in the present study, it is suggested that the progressive PRCL occurrence may have been circumvented in part. A possible explanation would be that the strategy of P4 device reinsertion was efficient to provide significantly superior plasma P4 concentrations during the presumed critical days of most potential malefic effects of PRCL, when comparing P4 concentration on the day before NSER in G-Control (4.0 ± 1.3 ng/mL) and G-P4 ewes (9.2 ± 2.8 ng/mL).

As well as device reinsertion in the present study led to a greater number of ova/zona pelucidae/embryos recovered compared with control group, in a recent study, ewes receiving 300 IU hCG on Day 4 after device removal showed superior plasma P4 concentration on the day before NSER (11.1 ng/mL) when compared to control ewes (6.9 ng/mL) (Dias et al., 2022), and consequently, the recovery rate was greater in treated sheep compared with control ones. Thus, possibly, the strategy of increasing P4 levels through an endogenous or exogenous way can be a valuable tool to increase embryo production in superovulated sheep.

Despite the significantly greater number of ova/zone pelucidae/embryos recovered in G-P4 compared to G-Control, no difference was seen in the number of viable embryos recovered, since the embryo viability rate was low in both groups. Fertilization failures are recurrent in multiple ovulation and embryo transfer programs in sheep (Evans and Armstrong, 1984). It can occur for several reasons, from poor oocyte quality to changes in the uterine and oviductal environment that led to interference in sperm transit (Armstrong, 1993). The problem may still have been potentiated in the present study because it was developed during the non-breeding season (21°19′ S latitude; Balaro et al., 2014), which may have reduced the reproductive capacity of males (Benmoula et al., 2017), as already shown by the difference in fertilization rate between breeding and non-breeding seasons in superovulated ewes (Okada et al., 2000).

Summarizing, the two most important effects of reinsertion of the P4 device during the early luteal phase in superovulated ewes were observed: (1) an increase of P4 concentration on the day before NSER, and (2) an increase in embryo yield. These positive effects can be cost-effective because the device was reinserted and reduced management when compared to three flunixin-meglumine

administrations, used to prevent PRCL (Figueira et al., 2020). This strategy reduces waste (syringes/needles) and the amount of P4 in the final device discarded, which means fewer environmental contaminants. Thus, intravaginal device reinsertion is more associated with the principles of the 3Rs (Russell and Burch, 1959) and green, clean, and ethical conserns (Martin and Kadokawa, 2006) in animal experimentation. Finally, we emphasize that more studies are needed to better detail the effects of reinsertion of the P4 device in the early luteal phase on ovarian activity, uterine environment, and specifically on embryos from superovulated donor ewes.

## 5. Conclusions

The reinsertion of the intravaginal P4 device increases the plasma P4 concentration on the day before NSER, leading to a greater number of structures recovered. This strategy can be considered as a tool to circumvent the potential negative effects of PRCL in superovulated ewes.

## CRediT authorship contribution statement

**M.E.F. Oliveira:** Methodology, Writing - review & editing. **T.D.B. Caldas:** Methodology, Investigation, Writing - review & editing. **J.N.D. Rodrigues:** Methodology, Investigation, Writing - review & editing. **G.B. Vergani**: Methodology, Investigation, Writing - review & editing. **L.V. Esteves:** Methodology, Investigation. **P.C.C. Rangel:** Statistical analyses. **J.M.G. Souza-Fabjan:** Methodology, Investigation, Writing - review & editing. **F.Z. Brandão:** Methodology, Investigation, Writing - review & editing. **J.F. Fonseca:** Conceptualization, Methodology, Resources, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal, or other relationships with other people or organizations.

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