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# Exogenous progestogen does not affect first-wave follicle populations and oocyte quality during ovarian stimulation with FSH in sheep

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ABSTRACT

The effect of short-term administration of medroxyprogesterone acetate (MPA) or natural progesterone ($P_4$) during ovarian stimulation with FSH on oocyte recovery was investigated in Santa Inês ewes. Ewes were treated with an intravaginal sponge containing MPA for 6 d; GnRH was applied 36 h after sponge removal and FSH was given in 3 injections (40, 24, and 16 mg, respectively) every 12 h after (D0, approximate ovulation time). At the first FSH dose, the ewes received either a new MPA sponge (n = 10) or a controlled device for internal release impregnated with $P_4$ (n = 10) or did not receive any device (n = 10). Ovarian dynamics were assessed every 12 h by transrectal ultrasonography from D-3 to D2. Oocytes were recovered by laparoscopic ovum pick-up (LOPU) on D2 and graded by morphologic quality. The number of small, medium, and large follicles at D0 and D2 (ultrasound examinations), number of both follicles aspirated and oocytes recovered at LOPU, recovery rate, and oocyte grade did not differ ($P > 0.05$) among treatments. Thus, the short-term use of MPA or $P_4$ during ovarian stimulation did not affect the first-wave follicle population or morphologic quality of oocytes. We would suggest that, in this protocol, the use of exogenous progestin is unnecessary.



## 1. Introduction

Progesterone ($P_4$) modulates various reproductive functions such as follicular growth and initial nutritional support of the embryo, and it blocks estrus expression and ovulation by actions in the hypothalamus. Because of this latter function, $P_4$ (natural progestogen) and its analog (synthetics progestogen) have been commonly used in estrus synchronization protocols and to block the LH surge during ovarian stimulation to allow oocyte recovery in donor ewes [1,2]. However, recent evidence has suggested that exogenous progestogen may have a deleterious effect on embryo quality, mainly when used in long-term treatment [1,2].

The "Day 0 protocol" was proposed to synchronize ovulation to embryo transfer programs [3,4]. It involves the application of a progestogen-releasing device for 6 d before the initiation of superovulation by FSH soon after normal ovulation; that is, during emergence of the first follicular wave [5,6]. Recent research demonstrates that the application of a controlled device for internal release (CIDR) that

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contained $P_4$ during FSH stimulation of the first wave of estrous cycle was able to improve embryo yield [7]. In addition, it was reported that under high $P_4$ concentrations, FSH stimulation before LOPU enhanced the quality of cumulus-oocyte complexes (COCs) and oocyte fertilization rate for in vitro embryo production in sheep [8]. However, a comparative effect of exogenous progestogen (natural and synthetics) used during ovarian stimulation with FSH on populations of follicles and morphologic quality of oocytes produced has not yet been demonstrated. Therefore, we developed the following hypotheses: (1) The use of exogenous progestogen during ovarian stimulation would not affect the population of follicles but would affect the quality of COCs, and (2) after estrus synchronization by the Day 0 protocol, when ovulation is confirmed, exogenous progestogen support may not be essential during ovarian stimulation for COC collection. Thus, this study aimed to investigate the effect of the synthetic (medroxyprogesterone acetate [MPA]) and natural ($P_4$) progestogen on first-wave populations of follicles and oocyte quality during ovarian stimulation of Santa Inês ewes.

## 2. Materials and methods

This study was approved by the Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol #721/2015).

### 2.1. Experiment location, animals, and design

This study was performed at Unipeco, Brazil (22°27′ S, 43°39′ W), in October 2017 (nonbreeding season). Thirty multiparous and clinically healthy Santa Inês ewes (mean ± SD: 3.9 ± 1.0 yr old, 51 ± 5.9 kg of body weight, and 3.2 ± 0.6 of body condition score/scale 0–5) were kept housed and received chopped elephant grass (*Pennisetum purpureum*) plus 200 g/animal of concentrate (12% crude protein) twice daily, water and mineralized salt ad libitum. All ewes had their estrus synchronized followed by implementation of the Day 0 protocol [5]. Our previous study [4] defined the best stimulatory protocol used in the present study, D0 was considered the approximate time of ovulation. Ovarian assessment was performed as described below by ultrasonography on D-3 to D2. Our experimental design is illustrated in Figure 1.

### 2.2. Estrus synchronization and ovarian stimulation

All ewes had their estrus synchronized by the use of intravaginal sponges containing 60 mg of MPA (Progespon; Schering Plow Animal Health, SP, Brazil) for 6 d. One day before sponge removal, 300 IU eCG (Novormon 5000; MSD Animal Health, SP, Brazil) and 0.12 mg cloprostenol sodium (Estron, Tecnopec, São Paulo, Brazil) were administered intramuscularly, as well as 0.025 mg lecirelin (Synthetic

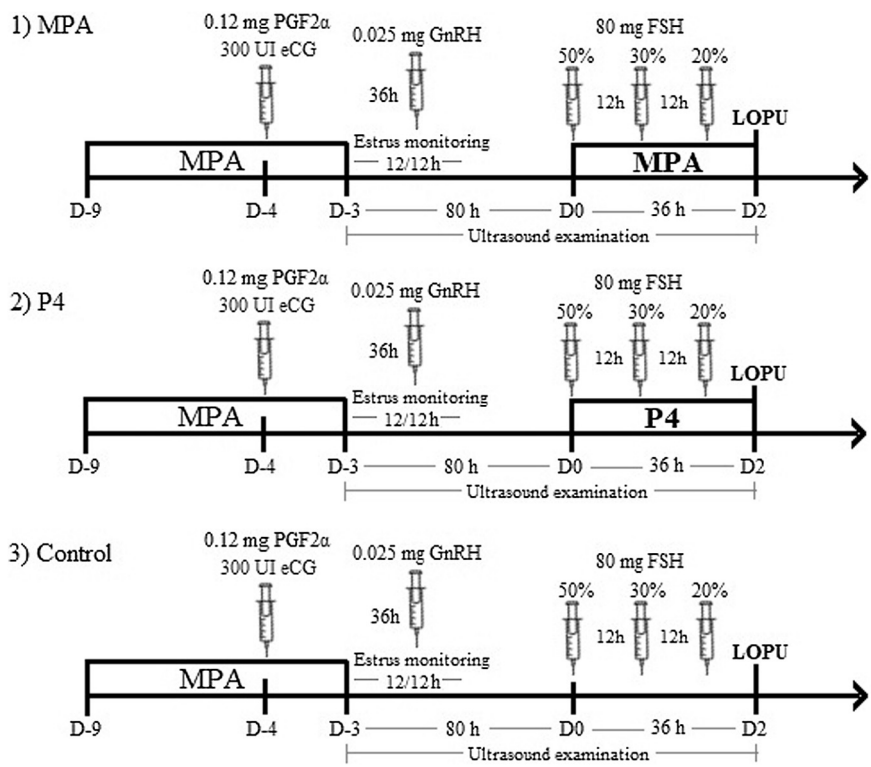


**Fig. 1.** Scheme of the experimental design. An estrus synchronization treatment was applied to all groups to synchronize ovulation on Day 0, when ovarian stimulation started, and a new progestin device was inserted that contained (1) medroxyprogesterone acetate (MPA), (2) natural progesterone ($P_4$), or (3) no device—control.

GnRH, Gestran Plus; Tecnopec, SP, Brazil) 36 h after sponge removal [5]. Ovarian stimulation started on D0, ewes received 80 mg of FSH (Folltropin-V; Bioniche Animal Health, Ontario, Canada) via 3 administrations (40, 24, and 16 mg, respectively) every 12 h.

### 2.3. Treatments

On D0, ewes in the progestogen-treated groups (n = 10 per group) received intravaginally either 60 mg of MPA (Progespon, Zoetis, São Paulo, Brazil), or a CIDR impregnated with 0.33 mg $P_4$ (Eazi-Breed CIDR, Zoetis). Control ewes (n = 10) did not receive any device (only luteal $P_4$ was thus present). The devices were removed immediately after LOPU (Fig. 1).

### 2.4. Estrous behavior, ovarian assessment, COC recovery, and grade

Estrous behavior was assessed using teaser rams (introduced every 12 h), and the female acceptance of mounting was considered decisive to confirm estrus, the first and last mounting defined the duration of estrus. B-mode transrectal ultrasonography (SonoScape, Shenzhen, China; 7.5-MHz linear transducer) was performed every 12 h from D-3 to D2. The ewes were restrained in a standing position for examination, and the procedure was carried out as described by Figueira et al [9]. Ovulation was characterized when the largest follicle visualized on the previous day was no longer observed and was also confirmed later by the presence of corpora lutea (CL) and their vascularization (functionality) by color Doppler mode. The population of follicles at Day 0 and Day 2 were used to compare the ovary status before and after treatments, respectively. Follicle diameters were classified as small (<3 mm), medium (3–5 mm), or large (>5 mm). At LOPU, all follicles between 2 and 8 mm were aspirated, and recovered COCs were graded according to morphologic features as G1 (multilayers of compacted cumulus and homogeneous ooplasm), G2 (1 to 3 layers and homogeneous ooplasm), G3 (1 incomplete layer or denuded but homogeneous ooplasm), and G4 (shapeless, expanded cumulus, and/or degenerated). G1 and G2 are considered to be good quality, G3 acceptable, and G4 poor quality [4].

### 2.5. Statistical analyses

Variables related to estrous behavior, ovulatory parameters, population of follicles, recovery rate, total number of COCs, and morphologic quality were tested for normality using the Lilliefors test. Parametric data were analyzed by ANOVA and Tukey test. Populations of follicles were analyzed, independently, for each day (D0 and D2), making comparisons of the follicular categories within each treatment and among treatments. Nonparametric data, as estrus response, was assessed by chi-square test. The SAEG 9.0 statistical analysis system program was used. Differences were considered significant when $P < 0.05$.

## 3. Results

### 3.1. Estrus synchronization and follicular wave

Overall, there were no differences among treatments in terms of estrous behavior and ovulatory parameters: estrous response rate [80% (range: 70%–90%), 24/30], initial sponge removal to onset of estrus (25.3 ± 2.4 h), initial sponge removal to ovulation (52.0 ± 2.4 h), estrus onset to ovulation (28.5 ± 2.6 h), and duration of estrus (35.3 ± 3.0 h). The ovulation rate (97% [29/30]), ovulations per ewe (1.3 ± 0.1), and the largest follicle diameter (6.7 ± 0.2 mm) also did not differ among treatment groups.

### 3.2. Populations of follicles and COC quality

Data on populations of follicles as determined by ultrasonography are shown in Table 1. On Day 0, no difference was observed among treatments in relation to the number of follicles in each category. In all groups, the number of small follicles was greater than that of medium follicles, which was greater than the number of large follicles. On Day 2, there was no effect of exogenous progestin on the number of follicles in any category. Regardless of the progestin source, the number of small and medium follicles was similar and, in turn, larger than the number of large follicles in all treatments. The variables observed during the LOPU procedure (follicles visualized and aspirated and number of COC recovered and recovery rate) and COC quality, as judged by morphological appearance, are shown

**Table 1**
Populations of follicles recorded by ultrasound examination immediately before (Day 0) and after (Day 2; day of LOPU) ovarian stimulation and initiation of variable treatments in hair ewes (mean per ewe ± SEM).

| Groups | Day 0 | | | Day 2 | | |
|---|---|---|---|---|---|---|
| | Small (<3 mm) | Medium (3–5 mm) | Large (>5 mm) | Small (<3 mm) | Medium (3–5 mm) | Large (>5 mm) |
| MPA | $8.6 \pm 1.1^{a,A}$ | $2.7 \pm 0.8^{a,B}$ | $0.1 \pm 0.1^{a,C}$ | $5.8 \pm 0.5^{aA}$ | $5.4 \pm 0.7^{aA}$ | $0.1 \pm 0.1^{a,B}$ |
| $P_4$ | $8.7 \pm 0.8^{a,A}$ | $2.0 \pm 0.5^{a,B}$ | $0.1 \pm 0.1^{a,C}$ | $4.8 \pm 1.0^{aA}$ | $5.2 \pm 0.7^{aA}$ | $0.6 \pm 0.3^{a,B}$ |
| Control | $8.8 \pm 0.8^{a,A}$ | $2.8 \pm 0.8^{a,B}$ | $0.2 \pm 0.1^{a,C}$ | $5.3 \pm 0.6^{aA}$ | $5.3 \pm 0.6^{aA}$ | $0.5 \pm 0.3^{a,B}$ |

Abbreviations: CIDR, controlled device for internal release; LOPU, laparoscopic ovum pick-up.
n = 10 ewes per treatment.
Within a column, a, b differ ($P < 0.05$) among the treatments, within the same day and follicular category.
Within a row, A, B differ ($P < 0.05$) among the follicular category, within the same day and treatment.
MPA: ewes received a sponge containing 60 mg of medroxyprogesterone acetate during stimulation.
P4: ewes received a CIDR containing 0.33 mg of progesterone ($P_4$) during stimulation.
Control: ewes did not receive any progestogen device during the stimulation.

**Table 2**
Total number of follicles visualized and aspirated by laparoscopy at Day 2, number of COC recovered and recovery rate, and quality of COCs using exogenous progestin during ovarian stimulation in hair ewes (mean per ewe ± SEM).

| Groups | Follicles | | COC | Recovery | [a]Good | [a]Acceptable | [a]Poor |
|---|---|---|---|---|---|---|---|
| | Visualized | Aspirated | Recovered | % | G1 + G2 | G3 | G4 |
| MPA | 10.1 ± 1.3 | 9.7 ± 1.3 | 5.9 ± 1.0 | 61 | 4.3 ± 0.9 | 1.4 ± 0.5 | 0.2 ± 0.1 |
| $P_4$ | 10.3 ± 0.8 | 10.3 ± 0.8 | 8.2 ± 0.7 | 80 | 5.3 ± 0.8 | 2.3 ± 0.8 | 0.5 ± 0.2 |
| Control | 9.9 ± 1.1 | 9.3 ± 1.1 | 6.2 ± 1.3 | 67 | 3.7 ± 0.7 | 2.0 ± 0.6 | 0.5 ± 0.2 |

Abbreviation: COC, cumulus-oocyte complex.
n = 10 ewes per treatment; ($P > 0.05$).
MPA: ewes received a sponge containing 60 mg of medroxyprogesterone acetate during stimulation.
P4: ewes received a CIDR containing 0.33 mg of progesterone ($P_4$) during stimulation.
Control: ewes did not receive any progestogen device during the stimulation.
[a] COC grading according to the cellular layers of cumulus and cytoplasmic uniformity: G1 (multilayered compacted cumulus and homogeneous ooplasm); G2 (1 to 3 layers and homogeneous ooplasm); G3 (1 incomplete layer or denuded but homogeneous ooplasm); G4 (shapeless, expanded cumulus, and degenerated) [4].

in Table 2. There was no difference among treatments for such variables.

## 4. Discussion

This study investigated for the first time the effect of 2 exogenous (natural or synthetic) progestogens versus endogenous $P_4$ (from CL; control) during ovarian stimulation with FSH on ovarian dynamics and oocyte production. The results demonstrated that exogenous progestogen does not affect the first-wave follicle population and oocyte morphologic quality in sheep.

Recently, it was reported that exogenous $P_4$ improved the number of grade 1 oocytes, despite the total number of COCs/ewe, and populations of follicles were not different between treated and nontreated ewes [8]. Regardless of the differences (FSH dose, administration regimen, and coasting time) between the present study and a study by Menchaca [8], our data do not corroborate those because we did not observe any differences, including on oocyte grade. In our previous study [4], 2 doses of FSH in one-shot or a multiple application regimen were tested. However, we only found an effect on the number of grade 2 oocytes in the multiple application regimen, regardless of the dose used [4]. Thus, this evidence suggests that the difference between the present study and a study by Menchaca et al [8] is perhaps related to the coasting time or FSH withdrawal (time between the last FSH administration and oocyte recovery). In cows, it was demonstrated that coasting time influences the developmental competence, but the COC morphologic grade was not mentioned [10]. These observations provide a new perspective for future research in hormonal protocols for oocyte production in sheep.

As expected, no differences in the estrous behavior or ovulatory parameters among experimental groups were observed after estrus synchronization because the same protocol was used to treat all ewes. These results corroborate those of our previous studies [4,5] demonstrating that the Day 0 protocol effectively synchronizes ovulation and the emergence of the first follicular wave, as previously reported [6]. The time chosen to initiate the stimulatory treatment was deemed appropriate based on the large number of small follicles and absence of large follicles [5].

On Day 0, the numbers of small, medium, and large follicles were similar across groups. The number of small follicles was greater than the number of medium follicles, and 85% of the ewes did not have large follicles, typically characteristic of the occurrence of a new wave of follicles postovulation. This finding is consistent with that of previous reports [5,6]. On Day 2, the population of follicles was not different among treatments. The number of small follicles decreased, and the number of medium follicles increased within each group compared with D0, an effect that is expected after ovarian stimulation [4].

In conclusion, short-term use of an exogenous progestogen during the ovarian stimulation protocol does not affect the population of first-wave follicles or morphologic quality of the COCs, thus rejecting our first hypothesis. On the other hand, it supports the hypothesis that when the occurrence of ovulation is confirmed, additional supply of progestogens by the use of exogenous devices is not required during the FSH treatment. We believe that luteal concentrations of $P_4$ are sufficient to prevent the LH surge and avoid oocyte maturation in vivo during the ovarian stimulation protocol.

## CRediT authorship contribution statement

**G.M. Bragança:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **J.M.G. Souza-Fabjan:** Conceptualization, Methodology, Formal analysis, Supervision, Visualization, Writing - review & editing. **L.S. Ribeiro:** Investigation, Data curation. **V.L. Brair:** Investigation, Data curation. **L.R. Côrtes:** Investigation, Data curation. **C.V. Souza:** Investigation, Data curation. **R.I.T.P. Batista:** Methodology, Writing - review & editing. **J.F. Fonseca:** Resources, Supervision, Writing - review & editing. **A. Menchaca:** Methodology, Writing - review & editing. **F.Z. Brandão:** Project administration, Supervision, Resources, Visualization, Conceptualization, Methodology, Writing - review & editing.

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

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