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Reuse of progesterone device did not affect embryo yield in locally adapted Brazilian sheep

[Reutilização de dispositivo de progesterona não afetou a produção de embriões em ovelhas brasileiras localmente adaptadas]

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Abstract: The reuse of progesterone devices is safe, efficient, and economically viable in small ruminants, supporting the evaluation of extended reuse protocols in superovulated ewes. Thus, the objective of this study was to test the efficiency of progesterone device reuse in superovulated ewes. Morada Nova (MN, n = 20) and Santa Inês (SI, n = 20) ewes were equally assigned to G-New (n = 20) and G-Used (n = 20) treatments. Ultrasound examinations in B- and Color Doppler modes were performed on D0 (device insertion), D7, D9 (device removal), D11, and D15 to assess ovarian dynamics. Superovulation consisted of six decreasing doses of p FSH (133 mg total) administered intramuscularly at 12 h intervals, starting 60 h before device removal. On D16, nonsurgical embryo recovery (NSER) was performed in ewes presenting at least one corpus luteum (CL). The number of follicles after p FSH treatment was greater ($P < 0.05$) in SI ewes. A breed-by-treatment interaction ($P < 0.05$) was observed for the interval to estrus, number of corpora lutea, and luteinized structures. Notably, a slight delay in estrus onset and poorer superovulatory responses occurred only in G-New MN ewes (60 % with CL and 4.7 ± 0.7 CL). However, they presented satisfactory numbers of CL and viable embryos per ewe flushed (7.8 ± 0.9 and 6.6 ± 1.1, respectively). The average total number of viable embryos in this trial was 9.2 ± 1.7. Positive correlations ($P < 0.05$) were found between ovarian blood perfusion on D15 and the number of luteinized structures/CL in G-New ($r = 0.79$) and G-Used ($r = 0.54$). However, correlations with recovered structures ($r = 0.63$) and viable embryos ($r = 0.63$) were observed only in G-New. In conclusion, reusing a progesterone device once for nine days (mid-term protocol) was as effective as using a new device in terms of embryo yield in locally adapted Brazilian ewes submitted to a superovulatory protocol and NSER.

Keywords: nonsurgical, ovine, reused, superovulation, ultrasound.

Resumo: A reutilização de dispositivos de progesterona é segura, eficiente e economicamente viável em pequenos ruminantes, o que respalda a avaliação de protocolos de reutilização estendida em ovelhas superovuladas. Assim, o objetivo deste estudo foi avaliar a eficiência da reutilização de

dispositivos de progesterona em ovelhas superovuladas. Ovelhas das raças Morada Nova (MN, n = 20) e Santa Inês (SI, n = 20) foram igualmente distribuídas nos tratamentos G-Novo (n = 20) e G-Usado (n = 20). Exames ultrassonográficos nos modos B e Doppler colorido foram realizados nos dias D0 (inserção do dispositivo), D7, D9 (remoção do dispositivo), D11 e D15 para avaliar a dinâmica ovariana. A superovulação consistiu na administração de seis doses decrescentes de p-FSH (133 mg no total), por via intramuscular, em intervalos de 12 h, iniciando-se 60 h antes da remoção do dispositivo. No D16, realizou-se a recuperação não cirúrgica de embriões (RNCE) nas ovelhas que apresentaram pelo menos um CL. O número de folículos após o tratamento com p-FSH foi maior ($p < 0,05$) na raça SI. Houve interação raça × tratamento ($p < 0,05$) para o intervalo até o estro, número de corpos lúteos e estruturas luteinizadas. Observou-se discreto atraso no início do estro e menor resposta superovulatória apenas nas ovelhas MN do grupo G-Novo (60 % com CL e 4,7 ± 0,7 CL), embora tenham apresentado números satisfatórios de CL e de embriões viáveis por ovelha submetida à lavagem uterina (7,8 ± 0,9 e 6,6 ± 1,1, respectivamente). O número médio total de embriões viáveis neste ensaio foi de 9,2 ± 1,7. Além disso, foram observadas correlações positivas ($p < 0,05$) entre a perfusão sanguínea ovariana no D15 e o número de estruturas luteinizadas/CL no G-Novo ($r = 0,79$) e no G-Usado ($r = 0,54$); contudo, apenas no G-Novo houve correlação com o número de estruturas recuperadas ($r = 0,63$) e de embriões viáveis ($r = 0,63$). Conclui-se que a reutilização do dispositivo de progesterona por nove dias (protocolo de médio prazo) apresentou eficácia equivalente à do dispositivo novo quanto à produção de embriões em ovelhas brasileiras localmente adaptadas submetidas a protocolo superovulatório e à recuperação não cirúrgica de embriões.

Palavras-chave: não cirúrgico, ovino, reutilizado, superovulação, ultrassom.

1. Introduction

Santa Inês (SI) and Morada Nova (MN) are locally adapted Brazilian sheep breeds raised under semi-arid Caatinga conditions and included in the Brazilian Conservation Germplasm Bank. These breeds represent valuable genetic resources for sustainable livestock production in challenging environments [1].

In vivo embryo production by superovulation has recently been performed in MN [2,4] and SI [3] breeds using a mid-term (9-day) protocol. The 9-day protocol developed in Lacaune ewes was based on the ovarian follicular pattern [5] prioritizing the initiation of the FSH regimen in the presence of a greater number of small follicles while avoiding larger ovarian follicles, as well as enabling stimulation of follicles from two ovarian waves [6]. Despite promising embryo yields, there are no reports describing the use of this protocol with the reuse of the progesterone device.

It has been demonstrated that a progesterone device previously used for 12 days can maintain luteal plasma progesterone concentrations for an additional six days in a short-term estrus induction protocol in acyclic goats [7] totaling 18 days of functional progesterone release. Similarly, in Santa Inês ewes, devices reused for a second time (10 days total) or third time (15 days total) in 5-day protocols maintained supraluteal progesterone concentrations and resulted in ovulation and fertility rates comparable to those obtained with new devices [8]. Considering that progesterone device reuse has been shown to be sanitary-safe in goats [7,9], efficient [8], and a beneficial and economically viable alternative in sheep [10], the 9-day protocol may provide an additional opportunity for device reuse for a further nine days, reinforcing the previously reported efficacy in MN [2,4] and SI ewes [3].

Material reuse represents an effective strategy for reducing input requirements and minimizing agricultural waste generation. This sustainable approach contributes to mitigating environmental impacts while aligning with clean, green, and ethical practices in animal agriculture [11]. Furthermore, although intravaginal devices represent a smaller cost component compared to FSH in superovulation protocols, as previously reported [6,12] their reuse may provide additional economic benefits that enhance the commercial viability of this biotechnology.

Although low progesterone concentrations during FSH superstimulation may favour embryo recovery [13], limited information is available regarding progesterone device reuse in mid-term (9-day) protocols for sheep superovulation. Addressing this gap is important because device reuse may reduce costs and environmental waste while maintaining efficiency, which is critical for the economic viability and sustainability of embryo production in conservation programs.

Therefore, this study aimed to compare the efficiency of new versus once-used progesterone devices for superovulation in Santa Inês and Morada Nova ewes.

2. Materials and methods

2.1 Ethical and animal conditions

This study was approved by the Ethics Committee for the Use of Animals of Embrapa Goats and Sheep (Process ID: 006/2016) and conducted from October to November in Sobral, Ceará State, Brazil (latitude 3°4′58.4″S and longitude 40°16′50.5″W).

Forty multiparous ewes of locally adapted Brazilian breeds (Santa Inês, n = 20; Morada Nova, n = 20) were selected. The animals had a mean body weight (BW)of 40.6 ± 2.1 kg (mean ± SEM), a body condition score of 3.3 ± 0.3 (scale 0–5), a mean age of 5.2 ± 0.2 years, and a mean parity number of 3.7 ± 0.9.

All females were maintained under an intensive production system with free access to corn silage, mineral salt, and water ad libitum. A balanced concentrate (200 g/animal) was provided twice daily.

2.2 Experimental design: estrus synchronization and superovulation in donor ewes

Ewes of both breeds were randomly assigned to two experimental groups and subjected to one of two estrus synchronization protocols, initiated in the evening on a random day of the estrous cycle (D0).

Each ewe received either a new intravaginal progesterone device (G-New; n = 10 Santa Inês ewes and n = 10 Morada Nova ewes) or a used device (G-Used; previously used once for nine days; n = 10 Santa Inês ewes and n = 10 Morada Nova ewes). The devices (0.33 g progesterone; CIDR® Eazi-Breed®, Zoetis, New Zealand) were maintained in situ for nine days.

All ewes received the superovulatory treatment starting 60 h before device removal, consisting of six decreasing doses (25 %, 25 %, 15 %, 15 %, 10 %, and 10 %) of p-FSH (133 mg total; Folltropin V®, Vetoquinol, Canada) administered i.m. at 12 h intervals. On Day 9, two equal doses of d-cloprostenol (37.5 µg; Prolise®, Agener União, Brazil) were administered i.m. concurrently with the fifth and sixth p-FSH injections.

All ewes also received three i.m. administrations of flunixin meglumine (2.25 mg/kg; Banamine®, MSD, São Paulo, Brazil) on Days 12, 13, and 15 (Figure 1).

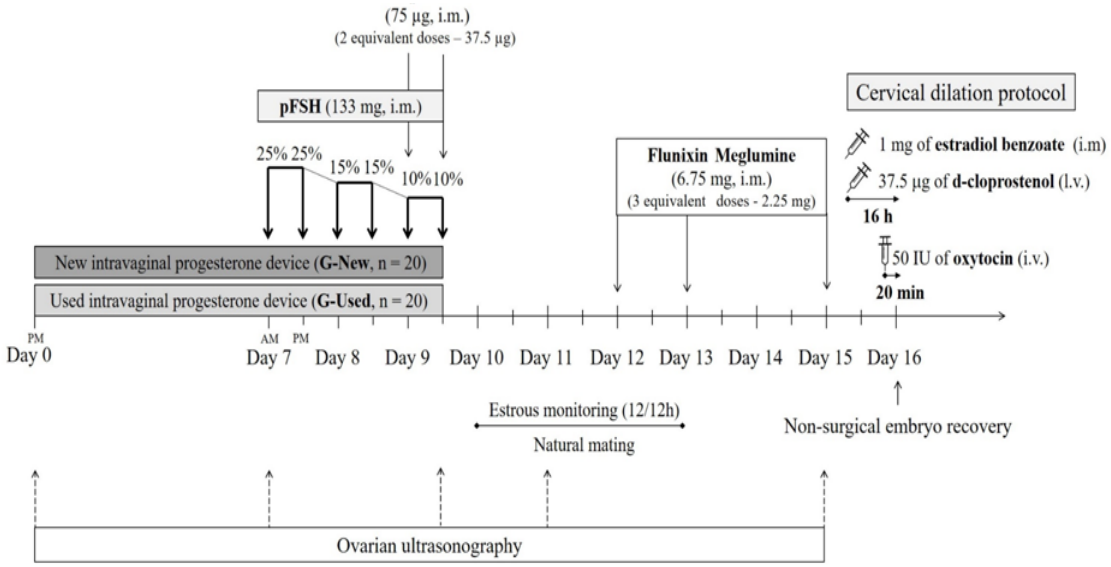

Figure 1. Schematic representation of the experimental procedures, including the estrus synchronization protocols followed by superovulatory treatment, and cervical dilation protocol in ewes subjected to nonsurgical embryo recovery (NSER) at 6.5 d after the device removal; pFSH: porcine follicle-stimulating hormone; i.m. intramuscular; i.v. intravenous; l.v.: latero-vulvar.

2.3 Estrus detection and natural mating

Ewes were kept with vasectomized rams equipped with ink-marked harnesses (ratio 10:1) and signs of estrus were observed twice daily (0800 and 1600) for 30 minutes, between 12 and 60 h after device removal. From the moment the females were first marked, they were mated three times at 12-hour intervals. Ewes were mated with rams (ratio 5:1) of the homologous breed, previously confirmed as fertile by breeding soundness examination. Rams underwent a comprehensive clinical examination with emphasis on reproductive tract evaluation. Libido was assessed during semen collection procedures and through behavioral observations. Semen quality was evaluated through mass motility (wave motion), progressive motility, sperm concentration, and morphological analysis of spermatozoa.

2.4 B- and Color Doppler mode ovarian ultrasonography

Ovarian ultrasonographic evaluations were performed on Days 0 (device insertion), 7 (first pFSH administration), 9 (sixth and final pFSH administration and device removal), 11 (36 h after device removal), and 15 (12 h before nonsurgical embryo recovery, NSER) (Figure 1). Examinations were conducted by an experienced technician using a Z5 Vet ultrasound system (Mindray®, Z5Vet, Digital Ultrasonic Diagnostic Imaging System, Brazil) equipped with a transrectal multifrequency (5–10 MHz) transducer. Standardized ultrasound settings were as follows: B-mode (depth 4.6 cm; gain 90 %; frame rate 55 frames/s; dynamic range 120 dB) and Color Doppler mode (frequency 5.7 MHz; gain 50 %; wall filter 183 Hz; pulse repetition frequency 1.1 kHz).

All visible antral follicles (≥ 2.0 mm) and detectable corpora lutea (CL) identified by B-mode ultrasonography were counted. Two perpendicular measurements were obtained to calculate the mean follicular diameters. Antral follicles were classified as small follicles (SF, ≤ 3.9 mm), medium follicles (MF, 4.0–5.9 mm), or large follicles (LF, ≥ 6.0 mm).

On Day 15, CL and luteinized anovulatory follicles (LAF) were quantified. LAF are defined as structures resulting from failed ovulation, measuring 5.0–10.0 mm in diameter [14]. The total number of luteinized structures (CL + LAF) was calculated because ultrasonographic examination alone

cannot definitively distinguish between ovulated and non-ovulated luteinized follicles without serial daily monitoring. This variable was considered an indicator of the total progesterone-producing capacity of the ovaries. The percentage of anovulatory luteinized follicles was calculated as (LAF/total luteinized structures × 100) to assess ovulatory efficiency in each experimental group.

Color Doppler ultrasonography was used to assess ovarian blood perfusion on Days 11 and 15. The mean percentage of ovarian blood perfusion area (Color Doppler signal area/ovarian cross-sectional area × 100) was calculated using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA), based on three consecutive frames centered around the strongest Doppler signal.

2.5 Nonsurgical embryo recovery

All ewes in which corpora lutea were detected by ultrasonography were submitted to uterine flushing for NSER on Day 16 (6.5–7 days after progesterone intravaginal device removal).

A hormonal protocol for cervical dilation was performed using d-cloprostenol (37.5 µg) administered I.v. and estradiol benzoate (1 mg; Estrogin®, Biofarm, SP, Brazil) administered i.m., both 16 h before NSER. Oxytocin (50 IU; Oxytocin Forte®, UCB, SP, Brazil) was administered i.v. 20 min before uterine flushing.

The NSER procedure was conducted as previously described [15,16]. Animals were subjected to the Embrapa analgesia and anesthesia protocol, which consisted of acepromazine maleate 1 % (0.1 mg/kg BW, i.m., 20 min before NSER; Aceproven®, Vencofarma, Londrina, Paraná, Brazil); dipyrone and *n*-butyl hyoscine bromide solution (5 mL i.v. and 5 mL i.m., 20 min before NSER; Buscofin Composto®, Agener União, Taboão da Serra, Brazil); and lidocaine 2 % (2 mL/animal epidural at S5–C1 and 5 mL intravaginally, administered 5 min and 3 min before NSER, respectively; Lidovet®, Bravet, Rio de Janeiro, Brazil).

The recovered fluid was filtered and examined under a stereomicroscope (Olympus SZ; Olympus Optical Co., Ltd., Tokyo, Japan) at 20–40× magnification. All retrieved structures were maintained in holding medium (Holding Plus, 0.4 % BSA; Embriocare, Cultilab, Brazil) and morphologically classified according to the criteria described in the 5th edition of the International Embryo Technology Society Manual [17]. Embryos classified as Grade 1 to 3 were considered viable.

2.6 Variables and statistical analysis

The following parameters were calculated: follicular populations on Days 0, 7, 9, and 11 (total number of antral follicles, small follicles, medium follicles, and large follicles; diameter in mm of the largest antral follicle); estrus response (number of females in estrus/number of treated females × 100); interval to estrus (interval in hours from device removal to first estrus detection); behavioral estrus duration (interval in hours from the first to the last estrus detection); estrus duration (interval in hours between the first and last acceptance of mounting); percentage of ewes with CL one day before NSER; ovarian blood perfusion area (%) on Days 11 and 15 (Color Doppler signal area/ovarian cross-sectional area × 100); number of CL on Day 15; number of luteinized anovulatory follicles (LAF) on Day 15; percentage of ewes successfully penetrated and flushed during NSER; number of CL per ewe flushed; number of recovered structures; and number of viable embryos. The recovery rate was calculated as (number of recovered structures/number of corpora lutea) × 100. When the number of recovered structures exceeded the number of CL

counted by ultrasonography, the recovery rate per ewe was capped at 100 %. The viability rate was calculated as (number of viable embryos / number of recovered structures) × 100. Ewes were also classified according to superovulatory response (< 10 CL or ≥ 10 CL one day before NSER) to evaluate the percentage of viable embryos obtained from different CL classes.

Data were analyzed using R software (Foundation for Statistical Computing, Vienna, Austria; Version 3.6.1, 2019). A 2 × 2 factorial arrangement was adopted in a completely randomized design (CRD) with time as a subdivided plot factor. Fixed effects included treatment, breed, day, and their interactions, according to the model proposed for a 2 × 2 factorial arrangement [18]. Variables related to estrus, superovulatory response, and embryo production were tested for normality using ANODEV. When global tests were significant, Tukey's test, chi-square test, or Fisher's exact test was used for pairwise comparisons, as appropriate. Longitudinal discrete data related to ovarian follicular populations were analyzed by ANODEV using generalized linear mixed models with negative binomial or Poisson distributions and a logarithmic link function. Interactions involving day were evaluated using ANOVA followed by Tukey's post hoc test for multiple comparisons. Pearson correlation coefficients were calculated between ovarian blood perfusion area (%) and estrus parameters, superovulatory response, and embryo production variables. Results are presented as least square means (LSMEANS) ± standard error of the mean (SEM). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 Ovarian population

Ovarian follicular populations recorded for the treatment groups at progesterone intravaginal device insertion (Day 0), first (Day 7) and last (Day 9) p-FSH administrations, and 36 h after device removal (Day 11) are presented in Figure 2. A significant interaction among the main effects (treatment, breed, and day of evaluation) was observed only for the total number of follicles, which was greater ($P < 0.05$) at 36 h after device removal in Santa Inês ewes. A main effect of treatment ($P < 0.05$) was detected for the total number of follicles (G-New: 11.1 ± 1.2; G-Used: 8.7 ± 0.9) and the number of small follicles (G-New: 9.0 ± 1.0; G-Used: 6.7 ± 0.6). A breed effect ($P < 0.05$) was observed for the total number of follicles (SI: 12.4 ± 1.3; MN: 7.4 ± 0.7), number of small follicles (SI: 9.7 ± 1.0; MN: 6.0 ± 0.5), and number of medium follicles (SI: 2.5 ± 0.5; MN: 1.2 ± 0.2). A significant day effect ($P < 0.05$) was found for the total number of follicles, number of small, medium, and large follicles, and diameter of the largest follicle, with the highest values observed on Day 11 compared to Day 0.

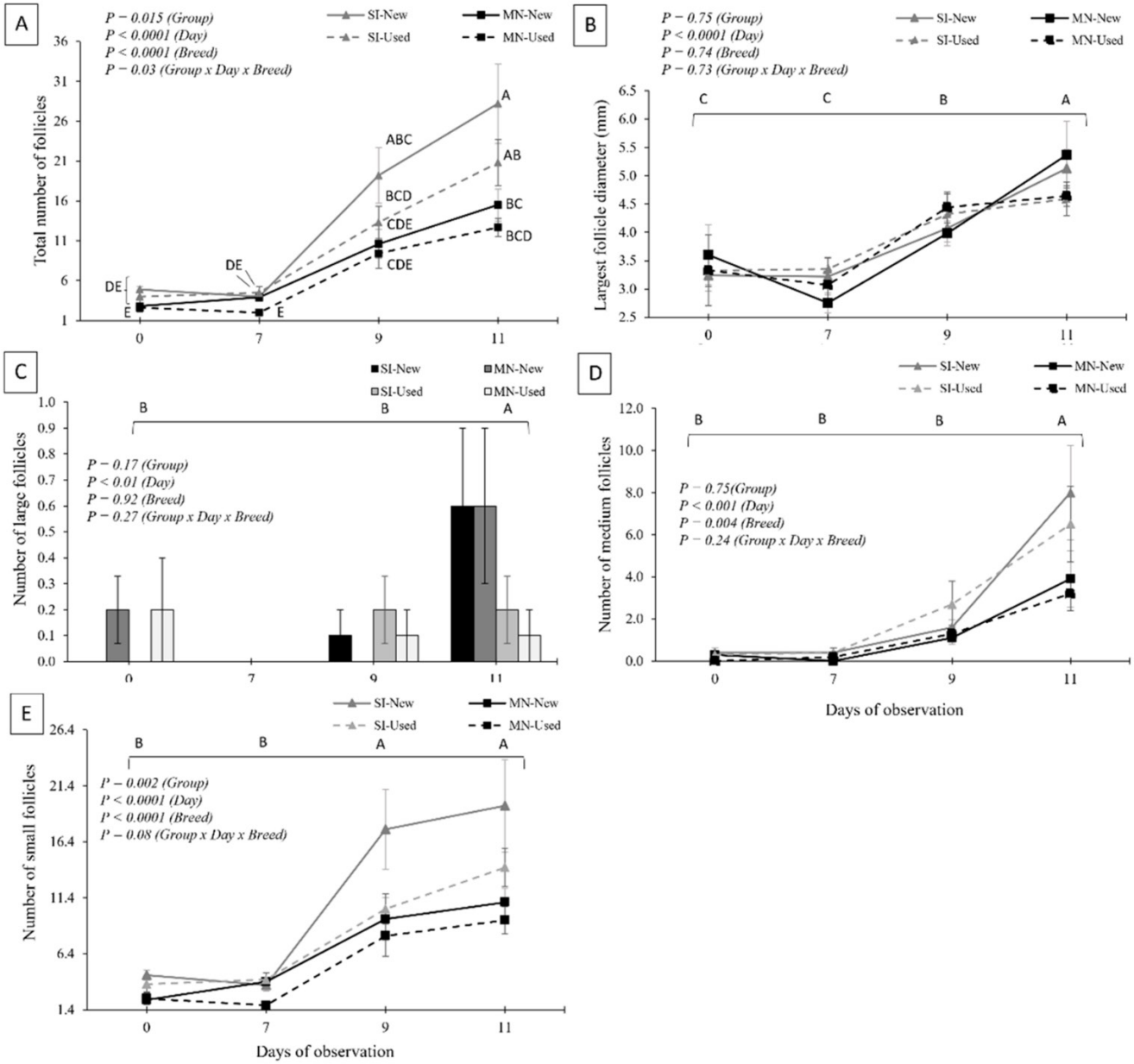

Figure 2. Ovarian follicular populations (mean ± SEM) at progesterone intravaginal device insertion (Day 0 p.m.), first (Day 7 a.m.) and last (Day 9 p.m.) pFSH administration, and 36 h after (Day 11 a.m.) intravaginal device removal of locally adapted Brazilian (SI: Santa Inês; and MN: Morada Nova) ewes submitted to 9-days estrus synchronization protocol with a new (New) or a used (Used; previously used once for nine days) intravaginal progesterone device associated to superovulatory treatment, and nonsurgical embryo recovery. ^{A,B}Different superscripts indicate statistical difference (P < 0.05) by Tukey's test.

3.2 Estrus, superovulatory response, and embryo recovery

Estrus behavior was not observed in any ewe at the time of device removal. The interval from device removal to estrus was less than 24 h in all groups, except in G-New MN ewes. Overall data related to estrus behavior, superovulatory response, and embryo recovery are presented in Table 1. NSER was successfully performed (i.e., successful cervical penetration and uterine flushing) in 93.7 % (15/16) of MN ewes and 89.5 % (17/19) of SI ewes.

Significant treatment × breed interactions were observed for interval to estrus, number of corpora lutea (CL), and total luteinized structures (P < 0.05). In G-New MN ewes, the interval to estrus was longer (P < 0.05) compared to G-Used MN ewes. Lower superovulatory responses, reflected by reduced numbers of CL and total luteinized structures, were also observed in G-New MN. In contrast, greater SOV responses were observed in G-New SI ewes. The number of CL per ewe flushed, the number of recovered structures, and the number of viable embryos did not differ

(P > 0.05) between treatment groups. The overall mean number of viable embryos per flushed ewe was 9.2 ± 1.7. The coefficient of variation for the number of viable embryos was 103 % overall (G-New MN: 92 %; G-New SI: 143 %; G-Used MN: 52 %; G-Used SI: 88 %).

Table 1 Estrus data, superovulatory response, and *in vivo* embryo production (LSMEANS ± SEM) of locally adapted Brazilian ewes submitted to a 9-day estrus synchronization protocol with a new (G-New) or a used (G-Used) intravaginal progesterone device associated with superovulatory treatment and nonsurgical embryo recovery.

Variables	Treatment				
	G-New		G-Used		
	Morada Nova	Santa Inês	Morada Nova	Santa Inês	
Estrus response (%)	100.0 [10/10]	100.0 [10/10]	100.0 [10/10]	90.0 [9/10]	0.99
Interval to estrus (h)*	25.0 ± 1.6^{A}	20.8 ± 1.4^{AB}	20.2 ± 1.4^{B}	23.0 ± 1.6^{AB}	0.03
Estrus duration (h)	20.4 ± 1.4	22.8 ± 1.5	21.6 ± 1.5	22.7 ± 1.6	0.65
Ewes with CL one day before NSER (%)	60.0 [6/10]	90.0 [9/10]	100.0 [10/10]	90.0 [9/10]	0.98
Corpora lutea count (*n*)	4.7 ± 0.7^{C} (0-18; 47)	13.8 ± 1.2^{A} (2-24; 138)	8.9 ± 0.9^{B} (3-12; 89)	11.8 ± 1.1^{AB} (0-27; 118)	<0.01
Luteinized anovulatory follicles	1.7 ± 0.4 (0-6; 17)	3.0 ± 0.5 (0-8; 30)	2.2 ± 0.5 (1-4; 22)	1.7 ± 0.4 (0-5; 17)	0.07
Total luteinized structures (*n*)	6.4 ± 0.8^{C} (0-20; 64)	16.8 ± 1.3^{A} (2-29; 168)	11.1 ± 1.1^{B} (6-15;111)	13.5 ± 1.2^{AB} (1-27, 135)	<0.01
% of anovulatory follicles	26.6 ± 5.5	17.9 ± 3.0	19.8 ± 3.8	12.6 ± 2.9	0.95
Ewes successfully penetrated and flushed at NSER (%)	83.3 [5/6]	90.0 [9/10]	100.0 [10/10]	88.9 [8/9]	0.98
Number of CL per ewe flushed (*n*)	7.8 ± 1.1 (4-18; 47)	13.7 ± 1.2 (2-24; 138)	8.9 ± 0.9 (3-12; 89)	13.1 ± 1.2 (5-27; 118)	0.42
Recovered structures per ewe flushed (*n*)	7.8 ± 1.2 (3-14; 39)	13.3 ± 1.2 (2-47; 120)	9.7 ± 1.0 (3-18; 97)	11.5 ± 1.2 (4-27; 92)	0.11
Recovery structures rate (%)	85.0 ± 13.7	64.0 ± 7.6	93.5 ± 10.7	91.8 ± 11.7	0.31
Viable embryos per ewe flushed (*n*)	6.6 ± 1.1 (0-14; 33)	10.8 ± 1.1 (0-42; 97)	8.8 ± 0.9 (3-16; 88)	9.6 ± 1.0 (0-27; 77)	0.11
Viability rate (%)	84.6 ± 5.7	80.8 ± 3.6	90.7 ± 2.9	83.7 ± 3.9	0.58
Ewes that had viable embryos recovered (%)	80.0 [4/5]	88.9 [8/9]	100.0 [10/10]	87.5 [7/8]	0.98
Ovarian blood perfusion on Day 11 (%)	10.3 ± 1.3	11.3 ± 2.6	6.4 ± 2.4	15.9 ± 2.2	0.65
Ovarian blood perfusion on Day 15 (%)	31.4 ± 4.6	28.4 ± 4.5	15.6 ± 6.4	31.4 ± 3.3	0.84

*Time from device removal to the onset of estrus. ( ) The range, lowest and highest observed values, and the total number are included in parentheses. [ ] The observed frequency was presented in square brackets. ^{A,B}Pairwise comparison by Tukey´s test, significant difference at P < 0.05.

Ewes with a CL count <10 before NSER contributed to a greater percentage (P<0.05) to the production of total embryos in the G-New group compared to G-Used (Figure 3).

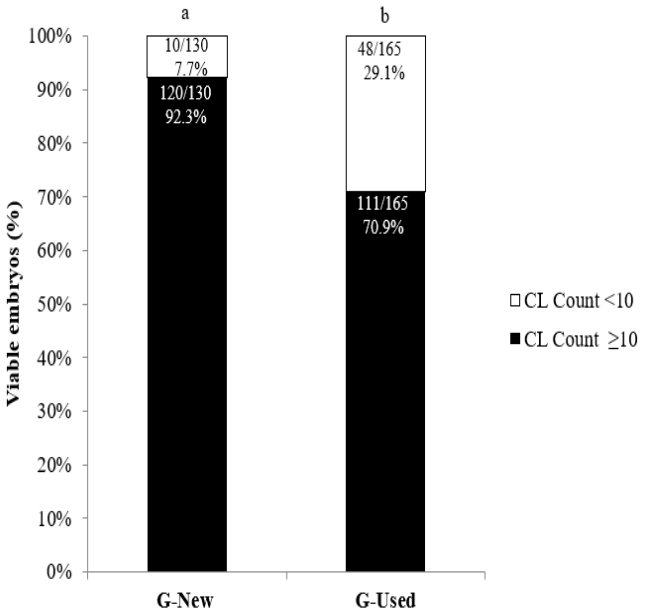

Figure. 3. Percentages of viable embryos after nonsurgical embryo recovery from Morada Nova and Santa Inês ewes with <10 or ≥10 corpora lutea (CL) that underwent a protocol of 9-days estrus synchronization with a new (G-New) or a used (G-Used) intravaginal progesterone device associated to superovulatory treatment. [A,B] Comparison by Chi-square test, significant difference at P < 0.05.

Significant positive and negative correlations (P < 0.05) were observed between ovarian blood perfusion percentage on Day 11 and estrus characteristics, superovulatory response, and embryo recovery variables only in the G-Used group. On Day 15, significant correlations were detected in both treatment groups (Table 2).

Table 2 Significant correlations of ovarian blood perfusion percentage on Day 11 and Day 15 with estrus responses, superovulatory responses, and embryo production of the locally adapted Brazilian ewes submitted to estrus synchronization protocol with a new (G-New) or a used (G-Used) intravaginal progesterone device associated with superovulatory treatment and nonsurgical embryo recovery.

	Input variable	Output variable	r	*P*- value
G-New	Ovarian blood perfusion percentage on Day 11	Interval to estrus*	-0.66	<0.01
		Estrus duration	0.48	0.03
		Total luteinized structures	0.78	<0.01
		Corpora lutea count	0.81	<0.01
		Number of viable embryos	0.57	0.03
	Ovarian blood perfusion percentage on Day 15	Corpora lutea count	0.81	0.01
		Total luteinized structures	0.79	0.01
		Number of recovered structures	0.63	0.01
		Number of viable embryos	0.63	0.01
G-Used	Ovarian blood perfusion percentage on Day 15	Interval to estrus*	-0.54	0.02
		Corpora lutea count	0.53	0.01
		Total luteinized structures	0.54	0.01

r-coefficient of correlation. *Time from device removal to the onset of estrus.

4. Discussion

Estrus behavior was observed in all females of the G-New group, irrespective of breed, and only one Santa Inês ewe in G-Used failed to display estrus signs. This high synchronization rate suggests that progesterone concentrations were maintained at levels sufficient to prevent spontaneous ovulation and promote synchronous estrus [9,19]. These findings are further supported by ultrasonographic monitoring of the ovarian follicular population. Although only slight differences in estrus behavior were observed between treatments, significant treatment × breed interactions were detected for superovulatory response, as reflected by total corpora lutea (CL) and total luteinized structures.

The total number of follicles was lower in Morada Nova compared to Santa Inês ewes. Breed effects are known to influence superovulatory responses [20], and some authors report that breed-related differences may account for approximately 30 % of the variability in embryo yield [21]. The antral follicular count (AFC) is one of the principal determinants of superovulatory response and embryo production in sheep and has been suggested as a criterion for donor selection, thereby avoiding stressful procedures in animals with limited superovulatory potential [22, 23].

Interestingly, 40 % of G-New Morada Nova ewes did not exhibit CL on ultrasonography performed prior to embryo recovery. The proportion of ewes with absent or limited ovulation represents a major source of variability in MOET programs, with reported incidences of approximately 20–30 % [24, 25]. One factor that may reduce ovulation rates is the presence of dominant follicles at the time of the first pFSH administration, as subordinate follicles may grow to preovulatory size but fail to ovulate [26].

The classification of large follicles (≥ 6.0 mm) was effective for confirming the absence of dominant follicles at treatment initiation (Day 7), which is critical for superovulatory success [24,25,26]. The presence of large follicles (≥ 6 mm) at FSH onset has been associated with reduced ovulatory responses [20,25,26]. However, this cutoff may be less appropriate for identifying preovulatory follicles on Day 11, when follicular maturation is advanced, and estrus is evident in most ewes. Other studies have defined large or preovulatory follicles using lower thresholds (≥ 5.0 mm) [4,27], which may provide greater sensitivity for detecting mature follicles. Nevertheless, this limitation did not affect the primary objective of comparing embryo yields between new and reused devices. Ovulatory failure and persistent anovulatory follicles are also reported even under the “Day 0 Protocol,” which is designed to initiate superstimulation in the absence of dominant follicles [27].

Embryo yield in the present study was greater than that reported in studies using similar protocols in Lacaune ewes [12] and/or comparable pFSH doses in Santa Inês ewes [28], despite lower reported percentages of LAF in those studies (7 % and 3.1–13.7 %, respectively). When satisfactory embryo yields (~7 viable embryos) are achieved, ovulatory failure rates of 15 % [6] and 30 % [29] have also been documented. In the present study, reuse of the progesterone device did not appear to influence the formation of anovulatory follicles.

In the G-New group, ovarian blood perfusion was a strong predictor of superovulatory response, correlating positively with CL number, total luteinized structures, and viable embryos on Days 11 and 15. In G-Used, moderate positive correlations were observed between CL count, total luteinized structures, and ovarian blood perfusion on Day 15. Moderate negative correlations were detected between the interval to estrus and ovarian blood perfusion on Day 11 (G-New) and Day 15 (G-Used).

Because correlation indicates association rather than causation, these findings must be interpreted cautiously. The greater variability in superovulatory responses observed in G-New may have contributed to the negative correlation observed in this group on Day 11. A rapid increase in estradiol secretion by preovulatory follicles would be expected to trigger estrus signs promptly, resulting in a shorter interval from device removal to estrus. Collectively, these data suggest that improved ovarian perfusion is associated with shorter estrus intervals, stronger superovulatory responses, and enhanced embryo production.

The observed correlations between ovarian blood perfusion and estrous behavior, superovulatory response, and embryo yield are consistent with previous studies evaluating the utility of Color Doppler ultrasonography for assessing luteal function [30] and embryo recovery potential [6,14,31]. Although CL counting using Color Doppler may slightly decrease in accuracy as CL number increases [32], the overall estimate remains clinically valuable. Ewes presenting ≥ 10 CL contribute approximately 90 % of total embryo production, whereas those with < 10 CL contribute only about 10 % [4]. Therefore, in cases of uncertainty in CL enumeration, assessment of total ovarian blood perfusion area—given its strong correlation with viable embryo numbers—may enhance decision-making regarding NSER through non-invasive ultrasonographic evaluation.

Mean viable embryo yield did not differ among treatment × breed groups. However, although not statistically significant, new progesterone devices yielded both the lowest and highest values, with the greatest coefficients of variation observed across genetic groups (Morada Nova and Santa Inês, respectively). Moreover, G-Used ewes with lower ovulatory responses (< 10 CL) contributed a higher proportion of viable embryos compared to G-New, suggesting more homogeneous responses. Under the conditions of the present study, device reuse appeared to reduce individual variability, a desirable characteristic in MOET programs [20].

In addition to supporting highly viable embryo production, the mid-term protocol effectively permitted reuse of intravaginal devices, totaling 18 days of device permanence. In commercial or experimental (cross-over) settings, donors are commonly superovulated twice at 30-day [12] to 60-day intervals [6]. Reuse of an intravaginal device within the same donor during sequential superovulatory protocols may therefore be feasible. Given the maintained embryo yields, device reuse represents a practical strategy for reducing superovulatory costs and minimizing environmental waste, aligning with Green, Clean, and Ethical principles for controlled reproduction in sheep [13].

This study is the first to report successful reuse of intravaginal progesterone devices in superovulated Santa Inês and Morada Nova sheep, two locally adapted Brazilian breeds. Reuse has previously been described in estrus synchronization protocols for Toggenburg goats after autoclaving [7,9] and in Santa Inês sheep following washing and air-drying [8]. In Toggenburg goats, devices used for 12 days were subsequently effective for an additional 6-day estrus induction protocol [7], indicating maintenance of plasma progesterone concentrations above 1 ng/mL for up to 18 days. In Santa Inês sheep, circulating progesterone concentrations did not differ between new and reused devices (used for ten days) from day 3 to the end of the 5-day synchronization protocol [8]. Despite differences in superovulatory response following p-FSH treatment, high embryo yields per flushed ewe were observed across treatments. Total AFC appears to be the principal factor underlying breed differences. Notably, device reuse reduced individual response variability while maintaining high embryo production efficiency.

5. Conclusion

Reuse of an intravaginal device previously used for nine days resulted in embryo yields comparable to those obtained with a new device, demonstrating that mid-term (9-day) protocols can effectively support the reuse of intravaginal progesterone devices for superovulation in ewes. When combined with appropriate FSH dosing and administration strategies, protocols using either new or reused devices elicited robust ovarian responses, which, together with efficient natural mating and NSER, resulted in high numbers of viable embryos recovered from locally adapted Brazilian sheep.

Conflict of interest statement

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

Data availability statement

The data will be made available upon request to the corresponding author.

Author contributions

Conceptualization: Lima, M. S. D., Teixeira, D. Í. A., Oliveira, M. E. F., Fonseca, J. F.; Formal analysis: Figueira, L. M., Lima, M. S. D., Vergani, G. B., Batista, R. I. T. P., Teixeira, D. Í. A., Oliveira, M. E. F.; Data curation: Figueira, L. M., Lima, M. S. D., Vergani, G. B., Batista, R. I. T. P., Fonseca, J. F.; Investigation: Lima, M. S. D., Vergani, G. B., Silva, K. M., Monteiro, A. W. U., Batista, R. I. T. P., Fonseca, J. F.; Methodology: Lima, M. S. D., Vergani, G. B., Batista, R. I. T. P., Teixeira, D. Í. A., Fonseca, J. F.; Project administration: Fonseca, J. F.; Writing: Lima, M. S. D., Fonseca, J. F., Figueira, L. M., Vergani, G. B., Silva, K. M., Monteiro, A. W. U., Batista, R. I. T. P., Teixeira, D. Í. A., Oliveira, M. E. F.

Generative AI use statement

The authors did not use generative artificial intelligence tools or technologies in creating or editing any part of this manuscript.

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