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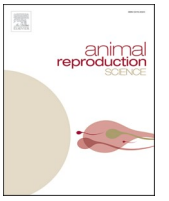

The effect of flunixin meglumine on the premature regression of corpus luteum, recovery rate, and embryo production in superovulated Dorper ewes

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ABSTRACT

This study evaluated the use of flunixin meglumine to prevent the occurrence of premature corpus luteum (CL) regression in superovulated ewes, improving embryo recovery and viability. Ewes (n=23) submitted to conventional superovulatory protocol and laparoscopic artificial insemination were treated with 2.2 mg/kg/day of flunixin meglumine (FLU, n=12) or 1.5 mL saline solution (CONT, n=11) on Days 2, 3, and 4 (Day 0 = 48 h after device removal). Serum progesterone (P_4) concentrations were measured (Day 1–6). Ultrasound (US, Days 3 and 6) and laparoscopic evaluation (Day 6) were performed to identify luteinized structures. In the US, laparoscopy, and P_4 assessments, the percentage of ewes with premature CL regression differed (P<0.05) between CONT (54.5; 63.6; and 54.5 %) and FLU (0.0; 0.0; and 0.0 %), respectively. The US exams revealed the effect (P<0.05) of treatment on the number of regressing CL between CONT (1.4 ± 0.6) and FLU (0.0 ± 0.0). Greater (P<0.05) number of normal CLs (10.5 ± 1.8 vs. 4.4 ± 1.5), ova/embryos (9.1 ± 2.1 vs. 3.7 ± 1.3), viable embryos (5.1 ± 1.1 vs. 2.6 ± 1.2), and recovery rate (79.5 ± 9.6 vs. 41.3 ± 15.0 %) were observed in FLU compared to CONT, respectively. The embryo viability rate did not differ (P>0.05) between FLU (60.7 ± 10.5 %) and CONT (45.5 ± 16.1 %). In conclusion, the flunixin meglumine protocol was able to prevent the occurrence of premature CL regression in superovulated ewes, increasing the recovery rate and embryo production.

1. Introduction

Among sheep breeds, Dorper stands out on the world stage in genetic improvement programs due to its adaptability to harsh

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environments, growth performance, and reproductive efficiency (Almeida et al., 2013; Souza et al., 2016; Kandiwa et al., 2020). Aiming at such programs, the multiple ovulation and embryo transfer (MOET) technique is essential to optimize the *in vivo* production of embryos from high-value females (Zhu et al., 2018; Bergstein-Galan et al., 2020). MOET was pointed out as the most used technique of ovine embryo production in Brazil, as well as in the world, and with a greater proportion if compared to the goat species (Souza-Fabjan et al., 2021).

Some obstacles, however, still prevent the best performance of the technique, such as the variation in the donor's ovulation rate, the number of viable structures per collection, and the occurrence of the premature corpus luteum (CL) regression, impairing its use on a commercial scale (Bruno-Galarraga et al., 2014; Bartlewski et al., 2016). Although premature CL regression was initially described and studied in superovulated goats (Armstrong et al., 1987; Battye et al., 1988; Saharrea et al., 1998), this condition was also verified in sheep (Schiewe et al., 1990), and continued until the present-day affecting females submitted to estrus-synchronization (Salloum and Saleh, 2022) and superovulation (Souza-Fabjan et al., 2017) in a percentage of 23 % and 60 %, respectively. In this context, our group has recently shown a premature CL regression rate of 25 % over four years of working performed with conventional superovulatory protocol in Dorper ewes, without finding any effect regarding the donor age, body condition score, number of MOET procedures, and season (Rocha et al., 2022). To the best of our knowledge, in most of the reports found in the literature, either no premature CL regression control strategy was applied to circumvent this problem in superovulated ewes, or no definitive results were established (Souza-Fabjan et al., 2017; Bergstein-Galan et al., 2020).

The mechanism of premature CL regression is frequently associated with high estradiol concentrations, often related to the presence of anovulatory follicles (Saharrea et al., 1998; reviewed by Rodriguez et al., 2015), resulting in an early release of prostaglandin F2 alpha (PGF2α). This event consequently decreases the concentration of progesterone (P_4), makes the environment unfavorable, impairing the embryonic migration in the oviduct and its viability (Saharrea et al., 1998; Aké-López et al., 2005; Cervantes et al., 2007). In superovulated goats, different approaches have been used to avoid the occurrence of premature CL regression, such as the use of hCG or GnRH (Saharrea et al., 1998), and P_4 or its analogs (Gilbert et al., 1990; Cervantes et al., 2007). Of note, flunixin meglumine, a non-steroidal anti-inflammatory drug that acts by inhibiting the synthesis of cyclooxygenase (the enzyme responsible for converting arachidonic acid to prostaglandins), appears to be an important strategy in goats over the years (Battye et al., 1988; Gilbert et al., 1990; Salles et al., 1998; Maia et al., 2020). In sheep, the flunixin meglumine was tested after mating, to check its effect on the length of the estrous cycle and luteal phase (Aké-López et al., 2005), but the literature is still incipient on the use of this drug to avoid premature CL regression in superovulated females and to achieve better results in embryonic production *in vivo* (Bergstein-Galan et al., 2020).

We hypothesized that the use of flunixin meglumine could minimize the occurrence of premature CL regression in superovulated ewes, increasing the production and recovery of good-quality embryos. Thus, the objective of this study was to verify the effects of flunixin meglumine on the functionality of the corpus luteum at the of embryo recovery, evaluating P_4 concentrations, macroscopic aspects of the corpus luteum by laparoscopy, and color Doppler ultrasonography images of ovaries, and consequently on embryo recovery and viability.

2. Material and methods

2.1. Ethics

This study was approved by the Animal Care Committee of the School of Veterinary Medicine and Animal Science of the University

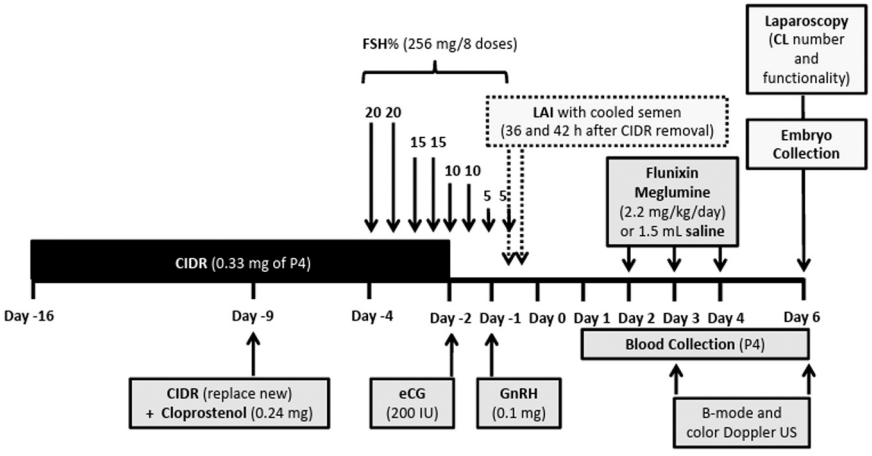

Fig. 1. Schematic representation of experimental procedures used to evaluate 2.2 mg/kg/day of flunixin meglumine (FLU) or 1.5 mL saline solution (CONT) after conventional superovulatory protocol and laparoscopic artificial insemination (LAI) on the functionality of corpus luteum (CL) and embryo production in Dorper ewes. CIDR: Controlled Internal Drug Release; eCG: equine chorionic gonadotropin; FSH: follicle-stimulating hormone; GnRH: gonadotropin-releasing hormone; P_4: progesterone; US: ultrasonography.

of São Paulo (protocol number: 2717181220).

2.2. Experimental location, animals, and design

The experiment was conducted at a commercial sheep farm in São Luis do Paraitinga, São Paulo State, Brazil (23° 22'36" S latitude and 45° 26' 51" W longitude). Multiparous Dorper ewes (n=23), previously approved in clinical evaluations, with ages between two and eight years (mean ± SEM of 3.2 ± 0.1), body weight of 64.2 ± 4.0 kg, and presenting good body condition score of 3.4 ± 0.2 (scale of 1–5) were studied. The animals were kept in a confined system, fed corn silage, and concentrated with about 10 % crude protein to meet maintenance requirements (National Research Council, 2007). Mineral salt and water were available *ad libitum*. The experimental design is shown in Fig. 1.

2.3. Superovulatory protocol, artificial insemination, and flunixin meglumine treatment

All ewes underwent the conventional superovulation protocol (Hameed et al., 2021). An intravaginal device containing 0.33 mg of P_4 (CIDR®, Zoetis, São Paulo, Brazil) was inserted on Day −16 and replaced by a new one on Day −9, along with 0.24 mg of cloprostenol (Sincrocio®, Ourofino, São Paulo, Brazil) via intramuscular (i.m). The superovulatory treatment with 256 mg of FSH (Folltropin®, Vetoquinol, São Paulo, Brazil) started on Day −4 divided into eight doses (20/20/15/15/10/10/5/5 %) administered i.m. every 12 h for four days plus 200 IU of eCG (Novormon®, Zoetis, São Paulo, Brazil) i.m. at P_4 device removal (Day −2) and 0.1 mg of GnRH (Fertagyl®, MSD, São Paulo, Brazil) i.m. on Day −1.

Laparoscopic artificial inseminations (LAI) were performed twice at 36 and 42 h after P_4 device removal, using commercially cooled semen from Dorper rams with proven fertility and tested according to seminal quality parameters (Colégio Brasileiro de Reprodução Animal CBRA, 2013). An inseminating dose at a concentration of 125 $\times 10^6$ spermatozoa was used in each uterine horn.

After the LAI the ewes were randomly allocated into two groups: (1) FLU (n=12), to receive i.m 2.2 mg/kg of flunixin meglumine (Flumax®, J.A. Saúde Animal, São Paulo, Brazil); or (2) CONT (control, n=11), to receive i.m. 1.5 mL saline solution. Both were administrated once a day for three days (Days 2, 3, and 4, being Day 0 = 48 h after P_4 device removal).

2.4. Ultrasonographic evaluation

B-mode and color Doppler US evaluations were performed on Day 3 and Day 6 by the same experienced operator using portable equipment provided with a 7.5 MHz linear transducer (Mindray Z5, Shenzen, China). Each ovary was first located using B-mode, followed by the activation of color Doppler mode to assess the presence or absence of blood perfusion, which was used as a marker of luteal function (Figueira et al., 2015; Bevilaqua et al., 2023). It quantified luteinized structures as corpus luteum (normal and regressing) and luteinized anovulatory follicles (LAF). The LAF was identified by their size ≥5.0 mm, the presence of a luteinized wall, and a cavity greater than 50 % of the diameter of the structure (Oliveira et al., 2018; Figueira et al., 2020). The Doppler settings used were: 1.0 kHz pulse repetition frequency, 7 cm depth, and 75 kHz wall filter.

2.5. Serum progesterone concentrations

Blood samples were daily collected in all ewes from Day 1 (72 h after P_4 device removal) to Day 6 (day of embryo recovery) to determine the serum P_4 concentrations. Samples were collected via jugular venipuncture into tubes with a clot activator (BD Vacutainer®, New Jersey, USA). Tubes were immediately placed on ice, transported to the laboratory, and centrifuged at 2000 × g for 15 min. Serum was removed, separated into aliquots, and stored at −20 °C for posterior determination of serum P_4 concentrations using a commercial solid-phase radioimmunoassay (RIA) kit (catalog #07–270105, MP Diagnostics Division; Orangeburg, NY, USA) in a single assay, in which the detection limit was 0.15 ng/mL. The standard curve provided by the kit was used, where the established points were as follows: 0, 0.15, 0.50, 1, 5, 20, and 80 ng/mL. Standard curve points were performed in duplicates. All samples were assayed in the same RIA to eliminate inter-assay variability. The intra-assay coefficient of variation was 9 %, and all data were within the minimum and maximum values of the curve.

Premature CL regression was considered to have occurred if P_4 concentrations decreased to <1 ng/mL on Day 6 (Saharrea et al., 1998; Okada et al., 2000). It is worth highlighting, however, that in the case of animals presenting simultaneously regressed and normal CL, and P_4 concentrations remaining above 1 ng/mL, the occurrence of premature CL regression was not considered (Oliveira et al., 2018).

2.6. Laparoscopy for CL count and surgical embryo recovery

Six days after LAI, the ewes were submitted to the embryo recovery procedure under general anesthesia (2–4 % isoflurane with 0–15 mL/kg/min of oxygen). For this, the females were previously deprived of food and water for 24 h and 12 h, respectively.

Immediately before the embryo recovery, CLs were enumerated according to their functionality classification by laparoscopy evaluation as normal (orange or bright red in color) or regressing (white or pale pink in color, reduced size), in addition to checking the presence or absence of LAF (Farin et al., 1986; Oliveira et al., 2018).

Only the females that presented three or more CLs, independent of the presence of premature CL regression were submitted to surgical embryo recovery via longitudinal ventral laparotomy. Briefly, after uterus exposure, an 18-gauge IV catheter (BD, New Jersey,

USA) was inserted near the utero-tubal junction, and the uterine lumen received an injection of 40 mL of warmed (37 °C) buffered phosphate solution (DMPBS, Biodux, São Paulo, Brazil) supplemented with 10 % adult bovine serum (Nutricell, São Paulo, Brazil). This flushing medium was recovered using a Foley catheter (size 10 Fr) inserted at the external bifurcation of each uterine horn. Flushing content was recovered in a Petri dish (150 ×20 mm) and sent for evaluation. Embryo morphology was evaluated under a stereomicroscope (Nikon SMZ800N, Tokyo, Japan) using magnification from 20 to 40x and following the same principles used for cattle of the International Embryo Transfer Society (IETS, Stringfellow and Givens, 2010); embryos Grade I, II, and III: viable, and Grade IV: degenerated.

2.7. Endpoints and statistical analysis

We determined the following end points: estrus response (number of ewes in estrus/number of treated ewes x 100); ewes that responded to superovulatory protocol (ewes that had ≥3 CLs at laparoscopy/number of ewes that underwent superovulatory protocol x 100); total number of CL at laparoscopy between regressed and normal; number of normal CL at laparoscopy; number of regressing CL at laparoscopy; percentage of ewes presenting LAF at laparoscopy; percentage of ewes with premature CL regression at US (number of ewes with premature CL regression at US/ number of ewes that responded to superovulatory protocol x 100); percentage of ewes with premature CL regression by laparoscopy (number of ewes with CL that regressed/ number of ewes that responded to superovulatory protocol x 100); percentage of ewes with premature CL regression by P_4 concentrations (number of ewes with P_4<1 ng/mL on the three days before embryo recovery/number of treated ewes x 100); number of LAF, number of normal and regressing CLs, total number of luteinized structures counted by US on Days 3 and 6; number of total ova/embryos recovered in the flushing content (oocytes, zona pellucida, degenerated and viable embryos); number of viable embryos (Grade I to III); number of nonfertilized oocytes; number of degenerated (Grade IV) embryos; recovery rate (recovered structures/total CL counted at laparoscopy x 100) and viability rate (viable embryos/total recovered structures x 100).

The statistical analysis was performed by generalized linear models using the SAS® software (Statistical Analysis Software, Cary, NC, United States of America). The binomial variables were expressed as percentages and analyzed using the GLIMMIX procedure, with the logit link function, and treatment as a fixed effect. The continuous data samples were tested for normality of residuals using the UNIVARIATE procedure (Shapiro-Wilk test) and transformed, when necessary, via PROC RANK. The homogeneity of variances was assessed using the Levene test. Data with normal distribution were analyzed by PROC GLM. The deviance analysis was used for parametric data with POISSON or GAMMA distribution, using the log link function and treatment as a fixed effect. US measurements were also analyzed considering the ewes group that suffered premature CL regression (i.e., P_4<1 ng/mL) in comparison with the ewes that did not suffer (i.e., P_4>1 ng/mL) on the day of embryo recovery (Day 6).

Data from P_4 concentrations and US assessment of total luteinized structures on Day 6 were analyzed as repeated measures using PROC GLIMMIX, with Gamma and Poisson distribution, respectively. The compound symmetry (CS) matrixes were applied to model the residual covariance. The model included treatment (or group), day, ewe (as a random variable), and interactions where necessary. The Tukey's test was used to pairwise multiple comparisons. It was considered 5 % of the significance level for all tests. Correlation coefficients between P_4 concentrations on Day 6 and measurements obtained by laparoscopy or US as the number of CL normal, number of regressing CL, total number of luteinized structures, number of ova/embryos recovered, number of regressing CL, and number of viable embryos were evaluated using the Pearson correlations method

Table 1
Estrus parameters, superovulation response, and ovarian laparoscopic and ultrasonographic assessments of Dorper ewes treated with three doses of 2.2 mg/kg flunixin meglumine (FLU) or 1.5 mL saline solution (CONT) once a day, starting 96 h after the removal of the progesterone device *. Data are expressed as percentages or mean ± SEM. Minimum and maximum values are presented in brackets, when applicable.

Endpoints	CONT (*n*=11)	FLU (*n*=12)	Total	*P-value*
Estrus response (%)	100.0 (11/11)	91.7 (11/12)	95.7 (22/23)	0.98
Ewes that responded to superovulation (%)[1]	100.0 (11/11)	91.7 (11/12)	95.7 (22/23)	0.98
Total number of CL at laparoscopy	9.0 ± 1.0 [5 – 15]	10.5 ± 1.8 [5–26]	9.8 ± 1.1 [5–26]	0.48
Number of CL normal at laparoscopy	4.4 ± 1.5[b] [0 –14]	10.5 ± 1.8[a] [5–26]	7.6 ± 1.4 [0–26]	0.02
Number of regressing CL at laparoscopy	4.6 ± 1.6[a] [0 – 14]	0.0 ± 0.0[b] [0 – 0]	2.2 ± 0.9 [0–14]	0.01
Ewes with LAF at laparoscopy (%)	72.7 (8/11)	63.6 (7/11)	68.2 (15/22)	0.42
Ewes with PRCL at laparoscopy (%)	63.6[a] (7/11)	0.0[b] (0/11)	31.8 (7/22)	0.01
Ewes with PRCL at ultrasonography (%)	54.5[a] (6/11)	0.0[b] (0/11)	27.3 (6/22)	0.01
Ewes with PRCL at progesterone concentration (%)	54.5[a] (6/11)	0.0[b] (0/11)	27.3 (6/22)	0.01

CL: Corpus Luteum; LAF: Luteinized Anovulatory Follicles; PRCL: Premature Regression of Corpus Luteum. PRCL was considered on Day 6 according to their morphology and color (small, light pink, or white) by laparoscopy, luteal blood perfusion by color Doppler ultrasonography, or by serum progesterone concentration (P_4<1 ng/mL).

*Ewes (n=23) received a conventional superovulatory protocol consisting of 14 days (from Day −16 to Day −2) of progesterone device (replaced by a new one on Day −9), and 256 mg FSH (in eight doses, every 12 h, from Day −4 to Day −1), plus 200 IU eCG at device removal (Day −2), and 0.1 mg GnRH on Day −1.

[ab]Different letters in the same row differ statistically (P<0.05)

[1]Superovulation response was considered when the number of CL≥3 was counted by laparoscopy performed immediately before embryo recovery (Day 6).

3. Results

Among the ewes (n=23) studied, one did not show estrus behavior but responded to the superovulatory protocol, and another one did not respond to the superovulatory protocol (<3 CLs), the latter being excluded from the embryo recovery. Of the 22 ewes that responded to the superovulation, one ewe from the CONT presented normal and regressing CLs simultaneously at laparoscopic evaluation. Since this ewe showed serum P_4 compatible with functional CLs on Day 6 (10.09 ng/mL), we decided not to consider the animal as affected by premature CL regression.

The percentage of ewes with premature CL regression in the assessments performed by the US (CONT: 54.5 vs. FLU: 0.0 %), laparoscopy (CONT: 63.6 vs. FLU: 0.0 %), and serum P_4 concentrations (CONT: 54.5 vs. FLU: 0.0 %) showed differences (P<0.05) between groups (Table 1).

According to the data of P_4, there was an effect of treatment (P<0.05) in the mean concentration in FLU (5.9 ± 0.9 ng/mL) and CONT (3.6 ± 0.7 ng/mL) during the evaluation period (Day 1 up to Day 6), with higher values at the Day 4 and Day 6. The effect of day (P<0.05) was also observed. The P_4 concentration increased during the observed period, with the lowest on Day 1 and maximum on Day 6 (Fig. 2A). Grouping the ewes with premature CL regression or NORMAL regarding the functionality of the CL, it can be noticed that the latter showed higher (P<0.05) values of P_4 from Day 3 to Day 6 when compared to ewes that had premature CL regression

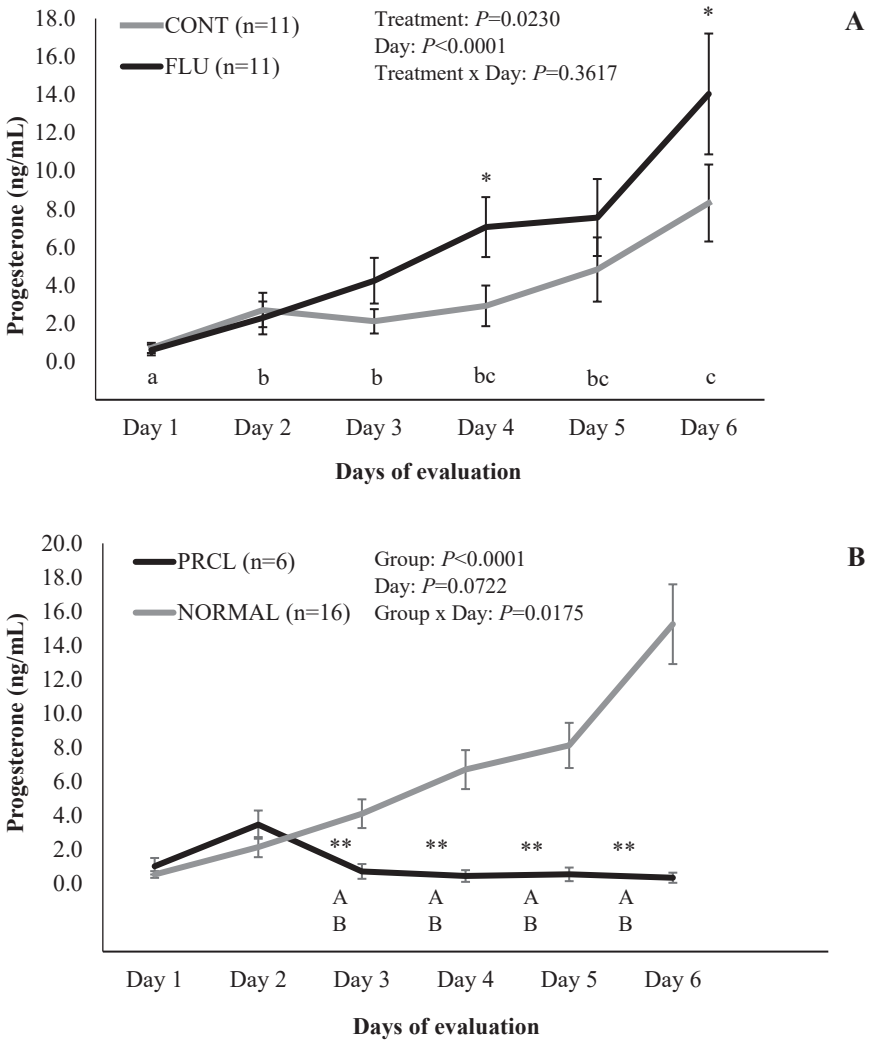

Fig. 2. Progesterone concentrations in Dorper ewes after traditional superovulatory protocol (intravaginal progesterone device for 14 days and 256 mg FSH, in eight doses), followed by treatment with flunixin meglumine or saline solution. Data are expressed as mean ± SEM. **Panel A:** The ewes of FLU were treated with three doses of 2.2 mg/kg flunixin meglumine and the ewes of CONT were treated with 1.5 mL saline solution on Days 2, 3 and 4. **Panel B**: The ewes with premature regression of corpus luteum (PRCL group, P_4<1.0 ng/mL on Day 6) were compared with ewes without PRCL (NORMAL group, P_4>1.0 ng/mL on Day 6).*Indicates the days in which differences between treatment groups were observed (P<0.05). ^{a,b,c} Different letters indicate differences between the days (P<0.01) ^{AB} Indicate there was an effect of the group with PRCL or without. **Indicate the interaction of the treatment per day, defined from Day 3 with the curves ascending in opposite directions (P<0.05).

(Fig. 2B).

Based on the US data performed on Day 3 and Day 6, it was observed effect of group ($P<0.05$) for the number of regressing CL (total) comparing CONT and FLU group (1.4 ± 0.6 and 0.0 ± 0.0), respectively (Table 2). In the comparison, also by the US, between the group of NORMAL ewes (i.e, without premature CL regression) and those that presented premature CL regression, there was group and day interaction, with an increase ($P<0.05$) in the number of luteinized structures from Day 3 to Day 6 in the NORMAL ewes (15.5 ± 1.3 vs. 18.9 ± 1.5, respectively), and, in contrast, a decrease ($P<0.05$) in these structures during the same days in the premature CL regression ewes (13.7 ± 2.9 vs. 8.7 ± 2.4, respectively). The number of luteinized structures on Day 6 (just before embryo recovery), therefore, was greater ($P<0.05$) in NORMAL ewes compared to the premature CL regression ones.

At laparoscopic assessment just before the embryo recovery, it was verified that the responsiveness of the ewes to the superovulatory protocol (≥3 CLs), the total number of CLs (normal and regressing), and the percentage of ewes presenting LAF were similar ($P>0.05$) between groups (Table 1). When evaluating the CL morphology, however, there was a greater ($P<0.05$) number of normal CL in FLU (10.5 ± 1.8 vs. 4.4 ± 1.5) and, in the opposite, a greater ($P<0.05$) number of regressing CL in CONT (4.6 ± 1.6 vs. 0.0 ± 0.0).

In one ewe from the CONT group, it was only possible to assess one ovary during the laparoscopy. Although this ewe was maintained in the study, the responses related to the ovarian observation, such as the numbers of ovarian structures and recovery rates, were not included.

The total number of recovered ova/embryos (9.1 ± 2.1 vs. 3.7 ± 1.3), viable embryos (5.1 ± 1.1 vs. 2.6 ± 1.2), and degenerated embryos (2.4 ± 1.5 vs. 0.1 ± 0.1) were greater ($P<0.05$) in ewes from the FLU when compared to CONT, respectively (Table 3). Likewise, the embryo recovery rate was superior ($P<0.05$) in FLU (79.5 ± 9.6 %) than in CONT (41.3 ± 15.0 %). The results referring to the number of nonfertilized oocytes (1.1 ± 0.6 vs. 0.7 ± 0.4), and embryonic viability rate (60.7 ± 10.5 vs. 45.5 ± 16.1 %), however, did not differ ($P>0.05$) between FLU and CONT groups, respectively. When grouping the ewes with premature CL regression (21.3 ± 11.3 %) and NORMAL ones (71.0 ± 10.5 %) it was noticed an inferior ($P<0.05$) recovery rate in the first group.

The serum P_4 concentrations on the day of embryo recovery were positively correlated ($P<0.05$) with the CL number (r=0.79), the total number of luteinized structures (r=0.51) by laparoscopy, and the number of ova/embryos recovered (r=0.54). Conversely, the P_4 concentrations were negatively correlated with the number of CL regressed (r=-0.58). There was no association ($P>0.05$) between P_4 concentrations and the number of viable embryos. Both CL normal counts by US or laparoscopy were positively correlated with the number of ova/embryos recovered (r=0.85 and 0.50, respectively) and the number of viable embryos (r=0.52 and r=0.51).

4. Discussion

The treatment with flunixin meglumine proposed by our group demonstrated that, in addition to being effective in preventing the occurrence of premature CL regression in Dorper ewes submitted to a conventional superovulatory protocol, it also resulted in a greater number of viable embryos and a greater recovery rate. In our study, the first administration of flunixin meglumine occurred approximately 54 h after LAI. It is worth mentioning that, on this day, the FLU group had five ewes with P4<1 ng/mL, while the CONT had only two. Conversely, on the day of embryo recovery (Day 6), there were no ewes from FLU with basal levels of P_4 compared to six from CONT. These data highlight the efficiency of flunixin meglumine treatment in increasing and maintaining adequate circulating P_4 levels. Playing a fundamental role in the initial period of embryonic development, including its transition from the oviduct to the uterus (Binelli et al., 2018), P_4 secreted by the CL is responsible for molecular and morphological modifications that occur in the reproductive tract environment (Rizos et al., 2010; Binelli et al., 2018), enabling the recovery of the embryo in the morula and blastocyst stages in the uterine horn on the day of collection. A positive correlation has already been reported between high concentrations of P_4 from immediately after conception through the 7th-day post-conception and the likelihood of embryo survival, development, and increased pregnancy rates (Forde et al., 2009). On the other hand, high estradiol concentrations resulting from

Table 2
Endpoints of ultrasonographic evaluation of luteinized structures on Day 3 and Day 6 in Dorper ewes submitted to superovulatory protocol, laparoscopic artificial insemination, and treated with 2.2 mg/kg/day of flunixin meglumine (FLU) or 1.5 mL saline solution (CONT) for three days starting 96 h after the removal of the progesterone device *. Data are expressed as mean ± SEM.

Endpoints	CONT (*n*=11)			FLU (*n*=11)			*P*-value		
	Day 3	Day 6	Total	Day 3	Day 6	Total	Trt	Day	Trt *Day
Number of LAF**	7.7 ± 1.5 (1 – 19)	5.0 ± 1.0 (0 – 9)	6.4 ± 0.9 (0 – 19)	8.8 ± 1.7 (2 – 19)	9.7 ± 2.2 (4 – 25)	9.2 ± 1.3 (2 – 25)	0.08	0.42	0.25
Number of CLs	7.0 ± 1.4 (0 – 17)	7.2 ± 2.1 (0 – 18)	7.1 ± 1.2 (0 – 18)	6.0 ± 1.4 (1 – 15)	7.9 ± 1.4 (3 – 15)	6.9 ± 1.0 (1 – 15)	0.82	0.69	0.37
Number of regressing CL	0.5 ± 0.3 (0 – 3)	2.4 ± 1.0 (0 – 11)	1.4 ± 0.6[a] (0 –11)	0.0 ± 0.0 (0 – 0)	0.0 ± 0.0 (0 – 0)	0.0 ± 0.0[b] (0 – 0)	0.01	0.09	0.09
Total number of luteinized structures	15.2 ± 1.7 (4 – 23)	14.5 ± 2.5 (2 – 26)	14.9 ± 1.5 (2 – 26)	14.8 ± 1.8 (6 – 24)	17.6 ± 2.0 (9 – 30)	16.1 ± 1.4 (6 – 30)	0.49	0.75	0.37

[a,b] Different letters in the same row differ statistically ($P<0.05$).

* Ewes received a conventional superovulatory protocol: 14 days (Day −16) of progesterone device (replaced by a new one on Day −9), and 256 mg FSH (decreasing doses every 12 h, from Day −4 to Day −1), plus 200 IU eCG at device removal (Day −2), and 0.1 mg GnRH on D-1.

** LAF: luteinized anovulatory follicles.

Table 3
Embryo recovery variables of Dorper ewes treated with three doses of 2.2 mg/kg flunixin meglumine (FLU) or 1.5 mL saline solution (CONT) once a day, starting 96 h after the removal of the progesterone device*. Data are expressed as a percentage or mean ± SEM. Minimum and maximum values are presented in parentheses when applicable.

Parameters	CONT (*n*=11)	FLU (*n*=11)	TOTAL	*P*-value
Number of total recovered ova/embryos	3.7 ± 1.3[b] (0–13)	9.1 ± 2.1[a] (0–26)	6.4 ± 1.4 (0–26)	<0.01
Recovery rate (%)	41.3 ± 15.0[b]	79.5 ± 9.6[a]	60.4 ± 9.6	<0.01
Number of viable embryos (Grade I to III)	2.6 ± 1.2 (0–11)	5.1 ± 1.1[a] (0–14)	3.9 ± 0.8 (0−14)	<0.01
Number of nonfertilized oocytes	0.7 ± 0.4 (0–4)	1.1 ± 0.6 (0–6)	0.9 ± 0.4 (0−6)	0.38
Number of degenerated embryos (Grade IV)	0.1 ± 0.1[b] (0–1)	2.4 ± 1.5[a] (0–16)	1.2 ± 0.8 (0–16)	<0.01
Viability rate (%)	45.5 ± 16.1	60.7 ± 10.5	53.9 ± 8.7	0.12

[a,b] Different letters in the same row differ statistically ($P<0.05$) *

premature CL regression increase motility in the female reproductive tract. This leads to the early migration of oocytes/embryos, placing them in an unsuitable environment, which negatively impacts the recovery rate (Souza-Fabjan et al., 2017). In this context, the flunixin meglumine treatment used in the present study successfully blocked premature CL regression. By maintaining high P_4 concentrations instead of high estradiol concentrations, observed in sheep and goats with premature CL regression, the treatment enabled the high number of structures recovered.

In the present study, the flunixin meglumine protocol tested (2.2 mg/kg administrated once a day for three consecutive days) was based on previous studies. To the best of our knowledge, however, results as satisfactory as ours have not been reported and, no standardized protocol has been effectively established. Battye et al. (1988) evaluated in superovulated goats, the twice-daily dosing of 2.2 mg/kg i.m. flunixin meglumine from Day 3 to Day 7 and reported that the P_4 level of goats treated rose from two days after the sponge removal (Day 0) and continued high until the end of the experiment. Conversely, the control goats presented a slight increase between Days 2 and 5 followed by a decrease in basal levels of P_4 on Day 6. With this study, the authors demonstrated the role of prostaglandin in early luteal insufficiency in superovulated goats. Ten years later, Salles et al. (1998) compared the use of 1.1 mg/kg (once or twice a day) and 2.2 mg/kg (once a day) for four days in goats and reported that the lower dosage, when administered twice a day, reduced the number of premature CL regression, however, differently from our results, the average number of recovered embryos and their viability did not differ between the treatments. Aké-López et al., (2005) used 2.2 mg/kg of flunixin meglumine twice a day, from Day 11–18 after natural mating, however, the objective of the study was to increase the lambing rate and prolificacy of ewes. The authors highlighted that the two variables studied were similar between the treated and the control ewes, and they concluded that the moment of flunixin meglumine application was late, probably after the beginning of the luteolytic process, leading only to a prolongation of the luteal phase and the estrous cycle. Bergstein-Galan et al. (2020) tested 1.1 mg/kg of flunixin meglumine in five administrations at 24-hour intervals. The authors reported no differences in the percentage of ewes with premature CL regression, embryo recovery, or pregnancy rates after embryo transfer. As unsatisfactory results were also verified by Salles et al. (1998), we can speculate that either the dosage was insufficient, or the frequency and day of administration were inadequate, since with our protocol it was possible to reach better results.

In addition to the data obtained by measuring P_4 concentration, the occurrence of premature CL regression was also verified in high percentages in the untreated group, by ultrasound and laparoscopy. Although it was not the main objective of this study, the methods of evaluation of premature CL regression used showed some equivalence, with important consequences for the decision-making of whether to harvest the embryos or not, in view of the high probability of low or null recovery of embryos and the reduction of the potential response after successive surgical recoveries. Interestingly, the US combining B-mode and color Doppler showed a similar correlation coefficient with the number of viable embryos compared to laparoscopic evaluation, probably due to the possibility of CL blood perfusion assessment. The use of this tool is reliable for selecting donors, minimizing interventions in animals with an insufficient response (Pinto et al., 2018; Figueira et al., 2020), and may serve in future works as a criterion to prioritize donors without premature CL regression on the day of recovery, since laparoscopy besides its invasiveness is performed just before the collection when the ewe has been already prepared for the procedure.

The US evaluation performed on Days 3 and 6 revealed a similar number of CL, LAF, and total luteinized structures in FLU and CONT. The number of regressing CL, however, differs ($P<0.05$) between treatments during the evaluated period. While the FLU group did not present any animals with premature CL regression, six animals in the CONT treatment underwent premature luteal regression as indicated by the drop in P_4 concentrations to baseline levels between Day 4 and Day 6. These animals had CL regressing on laparoscopy on Day 6, although one of them also had normal-appearing CL simultaneously in the same ovary at the same time, characterizing partial regression, as described by Saharrea et al. (1998). Data from the present study corroborate Okada et al. (2000), who suggest that premature CL regression in superovulated ewes may be due to the formation of abnormal CL with inadequate luteinization. According to Bevilaqua et al. (2023), the presence simultaneously of both normal and regressed CL in sheep, confirms that the early release of prostaglandin may not be the only mechanism related to premature CL regression. This phenomenon may also be associated with the individual characteristics of the animals concerning the pre-ovulatory follicles, CL development, and its responsiveness to luteolytic events. In the present study, the laparoscopy evaluation performed on Day 6 and the US assessments on Days 3 and 6, demonstrated, that the regressing CL was mostly found in the group of untreated ewes.

The need to find a strategy to overcome the abnormal CL function observed in ewes submitted to conventional superovulatory protocol followed by laparoscopic evaluation and embryo recovery was evidenced after finding a rate of ~25 % of premature CL regression in a four-year study conducted on the same sheep farm (Rocha et al., 2022). Thus, the result of 0 % premature CL regression

and good embryo yield obtained in the present study, demonstrated the feasibility and efficacy of the proposed protocol. Although the number of viable embryos collected was higher in the treated group (as expected), it was below the average of eight viable embryos per ewe obtained in previous collections by the same technician (Rocha et al., 2021). This fact may be related to the stress caused by the experiment when an increase in the number and handling of the animals by strangers and several simultaneous evaluations occurs. In these cases, there is a need for a period of adaptation to different conditions and even after this period, the animals may remain sensitive to small changes and stimuli (Pearson and Mellor, 1976). In stressful situations, the GnRH/LH pulse rate may be sufficient for follicular growth and development to occur. Interruptions or variations in pulse rate, however, can compromise granulosa cell integrity and oocyte quality. Although estrus and fertilization occur, the conceptus will not be competent enough to maintain the pregnancy and embryonic loss occurs (Dobson and Smith, 2000).

5. Conclusions

The flunixin meglumine was able to prevent the occurrence of premature CL regression in Dorper ewes after imposing a superovulatory regimen using pFSH and eCG, increasing the P_4 concentrations, recovery rate, and embryo yield. Considering the financial impact of premature CL regression and the low cost of treatment, the flunixin meglumine administration strategy is recommended as and valuable tool for improving the results of MOET programs.

CRediT authorship contribution statement

Joanna Maria Gonçalves Souza-Fabjan: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ana Lucia Rosa e Silva Maia:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lucas Machado Figueira:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marcela Sene Rocha:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Data curation, Conceptualization. **Jasmine Bantim de Souza Pinheiro:** Writing – review & editing, Methodology, Investigation, Data curation. **Thais de Almeida Oliveira:** Writing – review & editing, Methodology, Investigation, Data curation. **Felipe Zandonadi Brandão:** Writing – review & editing, Methodology, Investigation. **Jeferson Ferreira Fonseca:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Maria Emilia Franco Oliveira:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Claudio Alvarenga de Oliveira:** Writing – original draft, Visualization, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

Aké-López, R., Segura-Correa, J.C., Quintal-Franco, J., 2005. Effect of flunixin meglumine on the corpus luteum and possible prevention of embryonic loss in Pelibuey ewes. Small Rumin. Res. 59, 83–87. https://doi.org/10.1016/j.smallrumres.2004.11.008.

Almeida, A.M., Kilminster, T., Scanlon, T., Araújo, S.S., Milton, J., Oldham, C., Greeff, J.C., 2013. Assessing carcass and meat characteristics of Damara, Dorper and Australian Merino lambs under restricted feeding. Trop. Anim. Health Prod. 45, 1305–1311. https://doi.org/10.1007/s11250-013-0361-z.

Armstrong, D.T., Kiehm, D.J., Warnes, G.M., Seamark, R.F., 1987. Corpus luteum (CL) failure and embryonic loss in superovulated goats. Theriogenology 27, 207. https://doi.org/10.1016/0093-691X(87)90084-7 (abstract).

Bartlewski, P.M., Seaton, P., Oliveira, M.E.F., Kridili, R.T., Murawski, M., Schwarz, T., 2016. Intrinsic determinants and predictors of superovulatory yields in sheep circulating concentrations of reproductive hormones, ovarian status, and antral follicular blood flow. Theriogenology 86, 130–146. https://doi.org/10.1016/j.theriogenology.2016.04.024.

Battye, K.M., Fairclough, R.J., Cameron, A.W.N., Trounson, A.O., 1988. Evidence for prostaglandin involvement in early luteal regression of the superovulated nanny goat (*Capra hircus*). J. Reprod. Fert. 84, 425–430.

Bergstein-Galan, T.G., Weiss, R.R., Kozicki, L.E., Bortoleto, C.T., Lara, N.S.S., Aschenbrenner, G.A., 2020. Effect of flunixin meglumine and hCG at commercial programs for multiple ovulation and embryo transfer (MOET) in sheep. Arch. Vet. Sci. 25, 56–66. https://doi.org/10.5380/avs.v25i1.70477.

Bevilaqua, J.R., Rodriguez, M.G.K., Maciel, G.S., Vergani, G.B., Fonseca, J.F., Bartlewski, P.M., Oliveira, M.E.F., 2023. Luteal function, biometrics, and echotextural attributes in santa inês ewes superovulated with different total doses of porcine follicle-stimulating hormone. Animals 13, 873. https://doi.org/10.3390/ani13050873.

Binelli, M., Gonella-Diaza, A.M., Mesquita, F.S., Membrive, C.M.B., 2018. Sex steroid-mediated control of oviductal function in cattle. Biology 2, 7. https://doi.org/10.3390/biology7010015.

Bruno-Galarraga, M.M., Cueto, M., Gibbons, A.E., Pereyra-Bonnet, F., Catalano, R., Gonzalez-Bulnes, A., 2014. Repeatability of superovulatory response to successive FSH treatments in Merino sheep. Small Rumin. Res. 120, 84–89. https://doi.org/10.1016/j.smallrumres.2014.04.002.

Cervantes, M.J., Juaréz, M.L., Mejía, V.O., Berruecos, V.J.M., Vera, A.H., Valencia, J., 2007. Use of fluorogestone acetate after breeding to reduce the effect of premature luteal regression in dairy goats when superovulation is induced with FSH. Anim. Reprod. Sci. 97, 47–54. https://doi.org/10.1016/j.anireprosci.2006.01.008.

Colégio Brasileiro de Reprodução Animal (CBRA), 2013. Manual para exame andrológico e avaliação de sêmen animal, 3. ed. CBRA, Belo Horizonte, p. 87, 3. ed.

Dobson, H., Smith, R.F., 2000. What is stress, and how does it affect reproduction? Anim. Reprod. Sci. 60–61, 743–752. https://doi.org/10.1016/s0378-4320(00)00080-4.

Farin, C.E., Moeller, H.R., Sawyer, F., Gamboni, F., Niswender, G.D., 1986. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. *Biol. Reprod*, 35, 1299–1308. https://doi.org/10.1095/biolreprod35.5.1299.

Figueira, L.M., Fonseca, J.F., Arashiro, E.K.N., Souza-Fabjan, J.M.G., Ribeiro, A., Oba, E., Viana, J.H.M., Brandão, F.Z., 2015. Colour doppler ultrasonography as a tool to assess luteal function in Santa Inês Ewes. Reprod. Domest. Anim. 50, 643–650. https://doi.org/10.1111/rda.12543.

Figueira, L.M., Alves, N.G., Maia, A.L.R.S., Souza-Fabjan, J.M.G., Batista, R.I.T.P., Morais, M.C.D.C., Lima, R.R., Oliveira, M.E.F., Fonseca, J.F.D., 2020. Embryo yield and quality are associated with progestogen treatment during superovulation protocol in lactating Lacaune ewes. Theriogenology 155, 132–138. https://doi.org/10.1016/j.theriogenology.2020.06.004.

Forde, N., Carter, F., Fair, T., Crowe, M.A., Evans, A.C.O., Spencer, T.E., Bazer, F.W., McBride, R., Boland, M.P., O'Gaora, P., Lonergan, P., Roche, J.F., 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biol. Reprod. 81, 784–794. https://doi.org/10.1095/biolreprod.108.074336.

Gilbert, D.E., Conrood, S.A., Whiting, C.J., Pashen, R.L., 1990. Comparison of a progesterone intravaginal device (CIDR™) with flunixin meglumine (Finadyne™) for reducing the effects of corpora lutea regression in the goat. Theriogenology 33, 230 (abstract).

Hameed, N., Khan, M.I.-u-R., Zubair, M., Andrabi, S.M.H., 2021. Approaches of estrous synchronization in sheep: developments during the last two decades: a review. Trop. Anim. Health Prod. 53, 485. https://doi.org/10.1007/s11250-021-02932-8.

Kandiwa, E., Nguarambuka, U., Chitate, F., Samkange, A., Madzingira, O., Mbiri, P., 2020. Production performance of sheep and goat breeds at a farm in a semi-arid region of Namibia. Trop. Anim. Health. Prod. 52, 2621–2629. https://doi.org/10.1007/s11250-020-02283-w.

Maia, A.L.R.S., Arrais, A.M., Prellwitz, L., Batista, R.I.T.P., Figueira, L.M., Correia, L.F.L., Fonseca, J.F., Souza-Fabjan, J.M.G., 2020. Embryo development is impaired in goats that are treated for hydrometra and subsequently subjected to superovulation. Vet. Rec. 14 (187), e88 https://doi.org/:10.1136/vr.105906.

National Research Council (NRC), 2007. Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids. The National Academies Press, Washington, DC. https://doi.org/10.17226/11654.

Okada, A., Kamada, S., Jeon, C.-W., Miyamoto, A., Fukui, Y., 2000. Incidence of abnormal corpus luteum in superovulated ewes. J. Reprod. Dev. 46, 397–402. https://doi.org/10.1262/jrd.46.397.

Oliveira, M.E.F., Ribeiro, I.F., Rodriguez, M.G.K., Maciel, G.S., Fonseca, J.F., Brandao, F.Z., Bartlewski, P.M., 2018. Assessing the usefulness of B-mode and colour Doppler sonography, and measurements of circulating progesterone concentrations for determining ovarian responses in superovulated ewes. Reprod. Domest. Anim. 53, 742–750. https://doi.org/10.1111/rda.13165.

Pearson, R.A., Mellor, D.J., 1976. Some behavioural and physiological changes in pregnant goats and sheep during adaptation to laboratory conditions. Res. Vet. Sci. 20, 215–217.

Pinto, P.H.N, Bragança, G.M., Balaro, M.F.A, Arashiro, E.K.N, Santos, G.B., Souza, G.N., Souza-Fabjan, J.M.G., Fonseca, J.F., Brandão, F.Z., 2018. Colour-Doppler ultrasound imaging as a laparoscopy substitute to count corpora lutea in superovulated sheep. Reprod Domest Anim 53, 266e9. https://doi.org/10.1111/rda.13089.

Rocha, M.S., Pereira, P.V.S., Rangel, P.S.C., Valente, L.C.M., Oliveira, C.A., Souza-Fabjan, J.M.G., 2021. Res., Soc. Dev. 10, e43910918312 https://doi.org/10.33448/rsd-v10i9.18312.

Rocha, M.S., Maia, A.L.R.S., Rangel, P.S.C., Oliveira, M.E.F., Fonseca, J.F., Oliveira, C.A., Souza-Fabjan, J.M.G., 2022. Occurrence of premature regression of corpus luteum in MOET programs in Dorper ewes under subtropical climate. Livest. Sci. 255, 104808 https://doi.org/10.1016/j.livsci.2021.104808.

Rodriguez, M.G.K., Campanholi, S.P., Maciel, G.S., Oliveira, M.E.F., 2015. Premature luteal regression in small ruminants. Rev. Bras. Reprod. Anim. 39, 270–276 http://www.cbra.org.br/pages/publicacoes/rbra/v39n2/pag270-276%20(RB583).pdf (in portuguese).

Rizos, D., Carter, F., Besenfelder, U., Havlicek, V., Lonergan, P., 2010. Contribution of the female reproductive tract to low fertility in postpartum lactating dairy cows. J. Dairy Sci. 93, 1022–1029. https://doi.org/10.3168/jds.2009-2605.

Schiewe, M.C., Howard, J.G., Goodrowe, K.L., Stuart, L.D., Wildt, D.E., 1990. Human menopausal gonadotropin induces ovulation in sheep, but embryo recovery after prostaglandin F2alpha synchronization is compromised by premature luteal regression. Theriogenology 34, 469–486. https://doi.org/10.1016/0093-691x(90)90005-e.

Saharrea, A., Valencia, J., Balcázar, A., Mejía, O., Cerbon, J.L., Caballero, V., Zarco, L., 1998. Premature luteal regression in goats superovulated with PMSG: effect of hCG or GnRH administration during the early luteal phase. Theriogenology 50, 1039–1052. https://doi.org/10.1016/s0093-691x(98)00206-4.

Salles, H.O., Soares, A.T., Andrioli, A., Sobrinho, P.A.M., Azevedo, H.C., 1998. Different flunixin meglumine administrations to control of the corpus luteum premature regression in superovulated goats. Ciência Anim. 8, 69–74. ⟨https://www.alice.cnptia.embrapa.br/alice/bitstream/doc/515259/1/APIDiferentesposologias.PDF⟩ (in portuguese).

Salloum, A., Saleh, M., 2022. Comparison of GnRH and hCG effects on oestradiol, progesterone and premature luteolysis in Ovsynch-synchronized ewes. Reprod. Domest. Anim. 57, 550–555. https://doi.org/10.1111/rda.14094.

Souza-Fabjan, J.M.G., Rosa, R.M., Balaro, M.F.A., Pinto, P.H.N., dos Santos, G.B., Arashiro, E.K.N., Fonseca, J.F., Ungerfeld, R., Brandao, F.Z., 2017. Effect of different hormonal combinations on follicular wave emergence and superovulatory response in sheep. Theriogenology 103, 24–29. https://doi.org/10.1016/j.theriogenology.2017.07.036.

Souza, D.A., Selaive-Villarroel, A.B., Pereira, E.S., Silva, E.M.C., Oliveira, R.L., 2016. Effect of the Dorper breed on the performance, carcass and meat traits of lambs bred from Santa Inês sheep. Small Rumin. Res. 145, 76–80. https://doi.org/10.1016/j.smallrumres.2016.10.017.

Souza-Fabjan, J.M.G., Batista, R.I.T.P., Correia, L.F.L., Paramio, M.T., Fonseca, J.F., Freitas, V.J.F., Mermillod, P., 2021. *In vitro* production of small ruminant embryos: latest improvements and further research. Reprod. Fertil. Dev. 33, 31–54. https://doi.org/10.1071/RD20206.

Stringfellow, D.A., Givens, M.D., 2010. Manual of the International Embryo Transfer Society (IETS). fourth ed. Savoy, IL.

Zhu, J., Moawad, A.R., Wang, C.Y., Li, H.F., Ren, J.Y., Dai, Y.F., 2018. Advances in *in vitro* production of sheep embryos. Int. J. Vet. Sci. 6, 15–26. https://doi.org/10.1016/j.ijvsm.2018.02.00.