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Effects of different doses of estradiol benzoate used in a cervical relaxation protocol on the success of non-surgical embryo recovery and luteal function in superovulated ewes

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ABSTRACT

This study investigated the effectiveness of different doses of estradiol benzoate (EB) to promote cervical relaxation and their effects on luteal function and outcomes of non-surgical embryo recovery (NSER) in sheep. Multiparous (MULT) and nulliparous (NULL) crossbred Lacaune X Santa Inês ewes were superovulated and naturally bred. Seven days after progesterone withdrawal, females were randomly assigned to one of three distinct cervical relaxation protocols, consisting of i.m. treatment with 37.5 µg d-cloprostenol and different doses of EB: 0.0 mg (0.0EB group; n = 3 NULL and 14 MULT); 0.5 mg (0.5EB group; n = 4 NULL and 12 MULT) or 1.0 mg (1.0EB group, n = 6 NULL and 11 MULT) 16 h before NSER. All ewes received 50 IU of oxytocin 20 min before NSER (D17). Blood samples were collected and ultrasound exams (B-mode and color Doppler) were performed at two timepoints: immediately before d-cloprostenol and EB treatments and prior to NSER. Estrous behavior, corpora lutea count and NSER success outcomes were not affected by EB treatments nor parity ($P > 0.05$). Embryo recovery rate was greater for ewes in the 0.5EB group and in the NULL ewes ($P < 0.05$). Ovarian biometrics differed between the two evaluation timepoints in all groups ($P < 0.05$). Plasma estradiol increased over time, reaching a significant greater level in 1.0EB ewes compared to controls on D17 ($P < 0.05$), whereas progesterone concentrations decreased over time in all groups ($P > 0.05$). In conclusion, treatments did not affect NSER success but they did affect luteal function by altering P4 and E2 concentrations. Therefore, the NSER technique can be successfully performed in ewes with or without prior treatment with EB.

1. Introduction

Non-surgical embryo recovery (NSER) has been a great advance for the sheep embryo industry and animal welfare because it allows successive collections and the use of simpler anesthesia protocols [1]. Nevertheless, one of the

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major obstacles in popularizing the use of NSER in sheep is the need for a cervical relaxation treatment before embryo recovery. Several studies [1,2] have evaluated different treatment timing, routes, and drug dosage in an effort to develop a protocol that mimics the physiological cervical dilation mechanism, which naturally occurs during estrus and/or parturition [2]. This physiological mechanism involves the induction of changes in hormonal releasing patterns, mainly estradiol (E2) and prostaglandin E2 (PGE2) [3,4] which actions induce the lysis of the corpus luteum (CL) and, finally, the relaxation of the cervical tissue [5]. These two hormones act through a positive feedback mechanism, remodeling the cervical extracellular matrix and activating uterine oxytocin (OT) receptors, leading to a subsequent increase in uterine motility and contractility [2].

Considering the known effects of exogenous prostaglandin F2α (PGF) and estradiol benzoate (EB) on the mechanisms of cervical relaxation, it has been established a protocol based on treatment with these hormones 16 h before the NSER procedure attempting to activate OT receptors and induce cervical relaxation [1]. Thus, the combination of PGF and EB administered 16 h prior to and OT treatment 20 min before NSER has shown to benefit cervical transposition rates in goats and sheep [1,2,6]. Despite this convincing improve on NSER efficiency in sheep, transitory negative effects of EB on embryos recovered from superovulated sheep have been reported [7]. In addition, restrictions for the use of EB have increased in some countries [8]. Together, these limitations could prevent the adoption and expansion of the NSER technique worldwide.

In goats, EB treatment prior to NSER is not required and recent studies have shown up to 100% NSER success in this specie [9,10]. Interestingly, treatment with a PGF analogue (d-cloprostenol) 16 h prior to NSER in superovulated goats did not negatively affect pregnancy rates of fresh and frozen-thawed embryos transferred into recipient goats by semi-laparoscopy [11] or transcervically [12]. Taken that into account, our group previously tested and demonstrated the efficiency of no EB treatment for cervical relaxation in estrus-induced Dorper ewes, and reported interesting results that favors the reduction or exclusion of EB from the NSER protocol [13]. Nonetheless, as for other developing technologies, that previous study still needs to be replicated in superovulated donors. Another study reported a 10-fold increase in plasma E2 after EB treatment 16 h prior to NSER [14], but it is not known whether this increase was associated only to exogenous EB treatment or if PGF could also play a role in this E2 increase as well. The effects of different EB dosage in association with PGF in the cervical relaxation protocol prior to NSER, however, has not been determined in sheep. In particular, their impact on P4 and E2 profiles in superovulated ewes not receiving EB prior to NSER remains to be elucidated.

Considering the lack of knowledge about the effects of the EB-PGF based cervical relaxation protocol on ovarian function and circulating hormonal profile of superovulated ewes, the present study evaluated the feasibility of NSER in multiparous and nulliparous Lacaune x Santa Inês crossbred superovulated ewes subjected to a cervical relaxation treatment in the presence or absence of EB. Moreover, we tested whether a dose reduction and/or removal of EB from the protocol affect luteal function and the outcomes of the NSER technique.

2. Material and methods

2.1. Ethics, location, and experimental animals

This study was conducted after the approval by the Ethics in Animal Care Committee of Embrapa Dairy Cattle (protocol 6365271119). The experiment was carried out during the non-breeding season, at the experimental farm located in Coronel Pacheco, Minas Gerais, Brazil (latitude 21° 38′ 9′′ S, longitude 43° 19′ 9′′ W). A total of 36 multiparous (MULT) and 13 nulliparous (NULL) crossbred ewes (Lacaune X Santa Inês; n = 49), with 4.5 ± 0.2 and 2.5 ± 0.1 years of age (MULT and NULL, respectively) and 3.2 ± 0.1 and 3.1 ± 0.1 body condition score (scale 1 to 5; 1 = emaciated and 5 = obese) were enrolled in the study. All animals were kept in a confinement system, fed corn silage supplemented with a balanced ration and minerals with *ad libitum* access to water.

2.2. In vivo embryo production and NSER procedures

All ewes, at random stages of the estrous cycle, received an intravaginal device (D0) containing 0.36 g of P4 (PRIMER PR Caprinos e Ovinos, Tecnopec – Agener União, Taboão da Serra, Brazil) kept for nine days. Superovulation was induced by i.m. treatments with 333 IU of pFSH (PLUSET, Biogénesis Bagó, Curitiba, Brazil) beginning 60 h (D6) prior to P4 device withdrawal (D9). Ewes received pFSH twice daily (12 h intervals), in six decreasing doses (25%, 25%, 15%, 15%, 10%, 10% of the total dose), as depicted in Figure 1. Additionally, 37.5 µg of a PGF2α analogue (d-cloprostenol; Prolise, Agener União, Taboão da Serra, Brazil) was injected twice i.m., concurrently with the fifth and sixth doses of pFSH. After P4 device withdrawal, ewes were checked for estrous behavior and naturally mated to fertile rams (ram to ewe ratio 1:7) every 12 h, while in standing estrus.

The cervical relaxation protocol started 16 h before NSER. At this time, females were blocked by parity (MULT and NULL) and randomly assigned into three treatment groups to receive 37.5 µg of d-cloprostenol i.m. associated or not with different doses of EB (RIC-BE, Agener União, São Paulo, Brazil) as follows: 0.0 mg (0.0EB group; n = 16, NULL = 3 and MULT = 13); 0.5 mg (0.5EB group; n=16, NULL = 4 and MULT = 12) or 1.0 mg (1.0EB group, n=17, NULL = 6 and MULT = 11). All ewes were treated with 50 IU of oxytocin i.v. (Ocitocina Forte UCB, Jaboticabal, Brazil) 20 min before NSER.

The procedures for NSER were performed as previously described before [13]. Ewes that were not detected in estrus and/or did not present with a CL 24 h before NSER (n = 13) were indeed submitted to NSER to provide data on feasibility of the NSER technique, but other data from those females were not considered to evaluate superovulatory response nor the NSER procedure efficiency relative

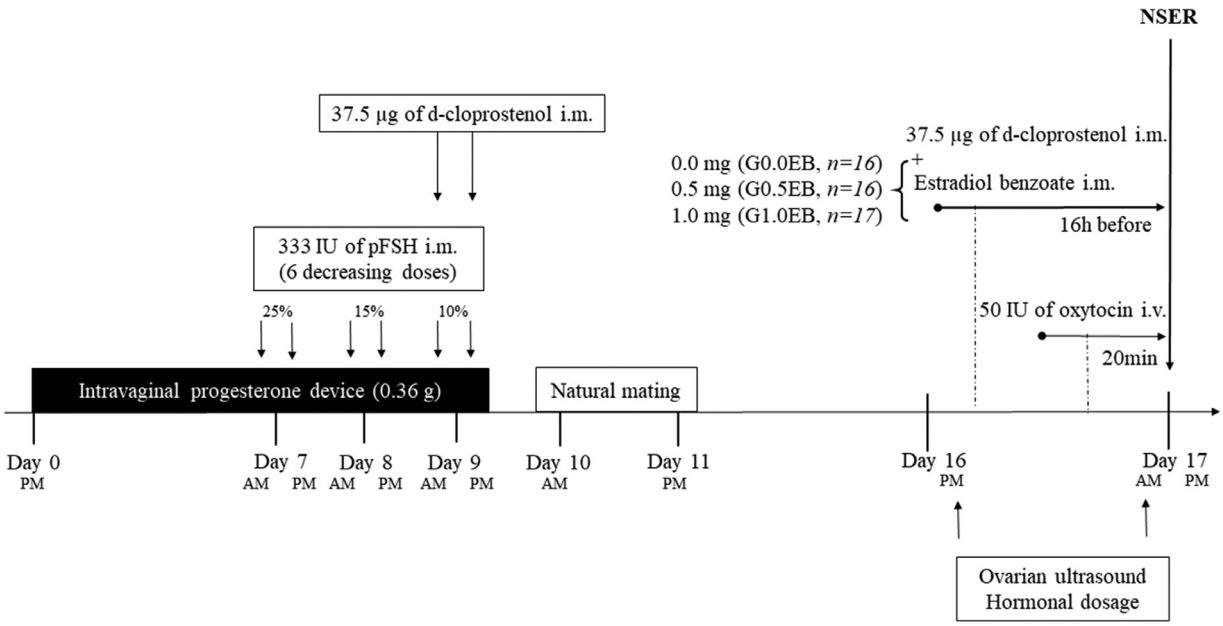

Fig. 1. Schematic representation of the experimental procedures performed to evaluate luteal characteristics, circulating E2 and P4 concentration profiles, and embryo yield in superovulated ewes submitted to a cervical relaxation protocol using different doses of EB (0.0, 0.5 or 1.0 mg) associated with PGF2α (d-cloprostenol) and oxytocin. i.m. intramuscular; i.v. intravenous prior to non-surgical embryo recovery (NSER).

to structures recovered. Ova/embryos recovered were classified according IETS manual [15] and recorded for all females.

2.3. Ultrasonography and image analysis

Ovarian ultrasound evaluations (B-mode and color Doppler) were performed immediately before the beginning of the cervical relaxation protocol (D16) and prior to the NSER procedure (D17) in all ewes using an ultrasound device (Mindray M5Vet, Shenzen, China) equipped with a transrectal linear probe, fitted with a rigid protractor (transducer frequency: 8.0 MHz; overall gain: 80%; depth 6.2 cm and dynamic range, DR: 70dB). The color Doppler settings were also the same during all evaluations (pulse repetition frequency, PRF: 0.9 KHz; overall gain 72%; depth 6.2 cm; transducer frequency: 4.6 MHz; and wall filter, WF: 1).

All antral follicles $\geq$ 5 mm in diameter, eventual luteinized anovulatory follicles (LUF), and CL present on the ovaries were counted, recorded, and measured using the Image J software (Wayne Rasband, National Institutes of Health, USA). The diameter (average length of the vertical and horizontal axes) and area of the CL were measured using the frame image at the largest CL diameter. Volume of luteal tissue was calculated using a formula for the volume of a sphere ($V = 4/3\pi r^3$, where r = radius and π = 3.1416). For CL with a fluid-filled cavity, the dimensions of the cavity were subtracted from total volume. Diameter, area, and volume of luteal tissue were calculated as the sum of all CLs on the ovaries of a given animal.

The area of ovarian blood perfusion was measured on a frame image containing the largest cross section of the ovary with the strongest color Doppler signals in addition to one frame immediately before and one frame immediately after, using the Image J software. The ovarian perfusion area (excluding the ovarian pedicle region) was calculated within each frame using the formula: *number of colored pixels /number of total ovarian pixels X 100*. Then, we calculated the average of the three evaluated frames. As for luteal echogenicity, we analyzed pixel brightness (mean pixel value; MPV) and pixel heterogeneity (standard deviation of the MPV) within a B-mode frame at the largest cross section of the ovary with the greatest number of visible luteal structures. Echogenicity was assessed using the Image Pro Plus 7.0TM software (Media Cybernetics Inc., San Diego, CA) with a scale of 256 shades of gray (0 = black and 256 = white). We used a selection tool to analyze circular areas of interest on the luteal tissue, avoiding eventual luteal cavities, if present.

2.4. Blood sampling and hormonal analysis

Blood samples were collected prior to ultrasound examinations (D16 and D17) by venipuncture of the jugular vein, using 4 mL vacuum tubes (BD Vacutainer Lithium Heparine Tubes, São Paulo, SP, Brazil). Samples were centrifuged at 1500 $\times$ *g* for 10 min, plasma was harvested and stored at -20°C until further analysis. Plasma progesterone and estradiol concentrations were determined by solid-phase radioimmunoassay (RIA) using I^{125} kits (MP Biomedicals LLC, Orangeburg, NY) according to the manufacturer's instructions. Sensitivity and intra-assay coefficients of variation were 5.0 pg/mL and 2.2% for estradiol and 0.075 ng/mL and 2.7% for progesterone, respectively.

Table 1
Superovulatory and non-surgical embryo recovery (NSER) outcomes (% or mean ± SEM) in ewes submitted to a superovulation protocol[d] and different doses (0.0, 0.5 and 1.0 mg) of estradiol benzoate plus 37.5 µg of d-cloprostenol 16 h before and 50 IU of oxytocin 20 min prior to NSER, used as a cervical relaxation protocol for NSER.

Endpoints	Estradiol benzoate			*P* value
	0.0 mg	0.5 mg	1.0 mg	
Estrus response (%)	93.7 (15/16)	93.7 (15/16)	88.2 (15/17)	0.80
Interval to estrus (h)	21.7 ± 3.0	24.0 ± 2.8	23. 3 ± 3.2	0.98
Ewes not responding to SOV (%)	33.3 (5/15)	20.0 (3/15)	6.7 (1/15)	0.24
Overall CL count[e]	7.0 ± 1.8 [105]	6.9 ± 1.2 [103]	8.6 ± 1.6 [129]	0.70
Overall LUF count[e]	2.0 ± 0.3 [18]	2.1 ± 0.3 [23]	2.5 ± 0.6 [28]	0.95
Ewes with LUF (%)	90.0 (9/10)	91.7 (11/12)	78.6 (11/14)	0.57
Successful Hegar dilator transposing rate (%)	100.0 (10/10)	83.3 (10/12)	71.4 (9/14)	0.09
Duration of Hegar dilator transposing (min)	2.4 ± 0.9	4.1 ± 1.2	4.5 ± 1.1	0.31
Duration of Mandrel-Catheter transposing (min)	1.6 ± 0.9	2.3 ± 1.0	1.2 ± 0.4	0.82
Duration of uterine flushing (min)	15.0 ± 0.7	20.2 ± 3.9	16.1 ± 0.9	0.20
Duration of NSER procedure (min)	20.4 ± 1.0	25.9 ± 3.3	22.2 ± 1.5	0.33
Successful NSER procedure rate (%)	90.0 (9/10)	83.3 (10/12)	64.3 (9/14)	0.28
Flushing efficiency (%)	100.0	100.0	100.0	-
CL count in ewes successfully flushed	10.1 ± 2.2 [91]	8.3 ± 1.1 [83]	10.8 ± 2.1 [97]	0.60
Recovery rate (%)	60.4[c] (55/91)	107.2[a] (89/83)	82.5[b] (80/97)	0.001
Ova/embryos recovered	6.1 ± 2.2 [55]	8.9 ± 1.7 [89]	8.9 ± 2.7 [80]	0.62
Transferable embryos[f]	3.7 ± 1.5 [33]	6.2 ± 1.7 [62]	4.8 ± 2.1 [43]	0.63
Unfertilized ova	1.9 ± 1.0 [17]	1.8 ± 1.2 [18]	3.7 ± 1.8 [33]	0.49
Degenerated embryos[f]	0.6 ± 0.3 [5]	0.7 ± 0.5 [7]	0.1 ± 0.1 [1]	0.72
Zona pelucidae	0.0 ± 0.0 [0]	0.2 ± 0.1 [2]	0.3 ± 0.2 [3]	0.77
Viability rate (%)	60.0 (33/55)	69.7 (62/89)	53.7 (43/80)	0.10

CL, corpora lutea; LUF, luteinized unovulated follicle.
(n/n) Number of animals, ovarian structures or recovered ova/embryos.
[n] Total sum of animals, ovarian structures or recovered ova/embryos.
[a,b,c] Within a row, means without a common superscript differed ($P < 0.05$).
[d] Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing doses of pFSH (333 IU, from 60 h before P4 device removal) and two doses of 37.5µg d-cloprostenol at the time of last two pFSH doses.
[e] Considering ewes that manifested estrous behavior with at least one CL on D16.
[f] Transferable embryos = embryos Code 1 and Code 2; Degenerated embryos = embryos Code 4.

2.5. Statistical analyses

The following data were recorded for all ewes enrolled in the study: number and proportion of ewes in standing estrus; interval from P4 removal to estrous behavior; number and proportion of SOV response (i.e. ewe in estrus with at least one CL on the day before NSER); overall CL count; overall LUF count; number and proportion of ewes presenting LUF on D16; time to pass the Hegar dilator through the cervix; number and proportion of ewes that the Hegar dilator was successfully passed through cervix; mean duration of mandrel-catheter passing through; duration of uterine flushing; duration of the NSER procedure; number and proportion of ewes successfully submitted to the NSER procedure (ewes successfully transposed with mandrel-catheter and flushed); flushing efficiency (percentage of fluid recovered post-infusion relative to the 400 mL of initial volume, calculated using the formula: volume retrieved / 400 mL x 100); CL count in ewes successfully flushed; ova/embryo recovery rate (considering ewes with at least one CL); number of ova/embryos recovered from ewes successfully flushed; number of transferable embryos (Code 1 and 2); number of unfertilized ova; number of non-viable embryos (Code 4); CL count on D16 and D17; luteal tissue diameter (mm), area (mm^2) and volume (mm^3); ovarian blood perfusion (mm^2); luteal tissue mean pixel value and heterogeneity; plasma P4 concentration (ng/mL); plasma E2 concentration (pg/mL).

Statistical analysis was performed using the SAS Studio software. The Bartlett test was used to determine homogeneity of variances and Shapiro-Wilk test used to verify normality. Parametric data (interval to estrus, CL count, time to pass Hegar, duration of mandrel passing, duration of flushing, duration of entire procedure, CL count in ewes successfully flushed) were analyzed by one-way ANOVA using the PROC GLM followed by a post-hoc Tukey test to determine differences among means. Non-parametric data (LUF count, number of ova/embryos recovered, transferable and degenerated embryos, unfertilized ova and zona pelucidae) were analyzed using PROC NPAR1WAY with the Wilcoxon test. For binomial data, contingency tables were analyzed using the Chi-Square test to determine statistical differences. Variables with repeated measures over time (plasma P4 and E2 concentrations, CL diameter, luteal tissue area and volume, ovarian blood perfusion and luteal tissue echogenicity and heterogeneity) were evaluated using the PROC MIXED procedure with a repeated statement to assess the interactions treatment and time. The statistical model included main effects of treatment, days of evaluation, and their interaction. Wilcoxon test was used to determine differences between means of ordinal-paired data. Statistical significance was determined based on a P-value of 0.05.

3. Results

Data relative to superovulatory responses and NSER outcomes are shown in Table 1. Table 2 presents superovulatory responses in NULL and MULT ewes. Overall, the 0.5EB group had a greater recovery rate compared with

Table 2
Superovulatory and non-surgical embryo recovery (NSER) outcomes (% or mean ± SEM) in superovulated[c] nulliparous and multiparous ewes submitted to a cervical relaxation protocol before NSER.

Endpoints	Category			*P* value
	Nulliparous	Multiparous	Total	
Estrus response (%)	92.3 (12/13)	91.7 (33/36)	91.8 (45/49)	0.60
Interval to estrus (h)	23.0 ± 2.7	26.8 ± 1.9	25.8 ± 1.6	0.65
Ewes not responding to SOV	0.0 (0/12)	27.3 (9/33)	20.0 (9/45)	0.13
Overall CL count[d]	8.2 ± 1.1 [98]	7.4 ± 1.2 [239]	7.6 ± 0.9 [337]	0.59
Overall LUF count[d]	1.9 ± 0.4 [23]	1.9 ± 0.3 [46]	1.9 ± 0.2 [69]	0.78
Ewes with LUF (%)	83.3 (10/12)	63.6 (21/33)	68.9 (31/45)	0.28
Successful Hegar dilator transposing rate	66.7 (8/12)	87.5 (21/24)	80.5 (29/36)	0.33
Duration of Hegar dilator transposing (min)	5.3 ± 1.2	3.1 ± 0.8	3.8 ± 0.7	0.31
Duration of Mandrel-Catheter transposing (min)	2.1 ± 1.2	1.6 ± 0.6	1.7 ± 0.5	0.79
Duration of uterine flushing (min)	21.1 ± 4.9	15.7 ± 0.5	17.2 ± 1.4	0.13
Duration of procedure	25.1 ± 4.2	21.1 ± 0.9	22.3 ± 1.4	0.60
Successful NSER procedure rate (%)	100.0 (8/8)	95.2 (20/21)	96.5 (28/29)	0.82
Flushing efficiency (%)	100.0	100.0	100.0	-
CL count in ewes washed	9.5 ± 1.0 [76]	9.7 ± 1.4 [195]	9.7 ± 1.0 [271]	0.91
Recovery rate (%)	107.9[a] (84/76)	71.7[b] (140/195)	82.6 (224/271)	0.001
Ova / embryos number	10.5 ± 2.7 [84]	7.0 ± 1.4 [140]	8.0 ± 1.2 [224]	0.27
Transferable embryos[e]	7.5 ± 1.3[a] [60]	3.9 ± 1.2[b] [78]	4.9 ± 1.1 [138]	0.05
Unfertilized ova	2.0 ± 1.9 [16]	2.6 ± 0.8 [52]	2.4 ± 0.8 [68]	0.55
Degenerated embryos	0.4 ± 0.4 [6]	0.3 ± 0.2 [7]	0.5 ± 0.2 [13]	0.92
Zona pelucidae	0.2 ± 0.3 [2]	0.1 ± 0.1 [3]	0.2 ± 0.1 [5]	0.90
Viability rate (%)	71.5[a] (60/84)	55.7[b] (78/140)	61.6 (138/224)	0.03

CL, corpora lutea; LUF, luteinized unovulated follicle.
() Number of animals, ovarian structures or recovered ova/embryos.
[] Total sum of animals, ovarian structures or recovered ova/embryos.
[a,b] Different letters indicate differences among groups (P < 0.05).
[c] Intravaginal device (0.36 g of progesterone) for nine days plus six decreasing doses of pFSH (333 IU, from 60 h before P4 device removal) and two doses of 37.5μg d-cloprostenol at the time of last two pFSH doses.
[d] Considering ewes that manifested estrous behavior with at least one CL on D16
[e] Transferable embryos = Code 1 and 2; Degenerated embryos = embryos Code 4.

groups 0.0 and 1.0EB ($P < 0.05$), whereas NULL ewes presented higher recovery and embryo viability rates compared with MULT ewes ($P < 0.05$). Three animals in 0.5EB group and one animal in 1.0EB group had an individual embryo recovery rate greater than 100% because the number of recovered structures was greater than CL count. Interestingly, these animals were all nulliparous. As a result, total recovery rate was increased in 0.5EB group and in the nulliparous animals. Time to complete the NSER procedure did not differ among groups nor parity ($P > 0.05$). The successful cervical transposing rate and the degree of difficulty using the Hegar dilator versus mandrel-catheter device did not differ among groups (Fig. 2; $P > 0.05$). In addition, we observed a greater number of animals classified into scores 1 and 2 for cervical transposing with Hegar or mandrel-catheter ($P < 0.05$).

Luteal morphology and functional data are shown in Table 3. We did not observe any differences in luteal morphology among groups on D16 and D17 ($P > 0.05$). Luteal diameter, volume, and area decreased from D16 to D17 in all groups ($P < 0.05$). The percentage of ovarian blood perfusion decreased from D16 to D17 ($P < 0.05$) only in the 0.5EB group. Plasma estradiol concentrations increased from D16 to D17 in all groups (Fig. 3A; $P < 0.05$). Within a day, E2 concentrations did not differ among groups on Day 16, whereas it was greater in 1.0EB group compared with 0.0EB group on D17 ($P < 0.05$). Plasma progesterone concentrations (Fig. 3B) decreased from D16 to D17 in all groups ($P < 0.05$) Table 3.

4. Discussion

This study confirmed that NSER can be successfully performed in superovulated Lacaune x Santa Inês crossbred ewes using a reduced EB dose or even abolishing the use of EB in the cervical relaxation protocol. In addition, this study demonstrates for the first time the feasibility of NSER in nulliparous animals, without affecting procedure duration nor its success rates. We provide evidence that not only EB but also PGF (d-cloprostenol) used to relax the cervix may affect luteal function of superovulated females submitted to NSER, mainly by triggering the process of luteolysis. Another important finding was that treatment with 1.0 mg of EB led to a 2.5-fold increase in plasma E2 concentration compared with 0.0EB group together with nearly basal levels of P4 in a phase of the estrous cycle (diestrus) where plasma P4 levels should be at/or close to their zenith. Therefore, this study brings novel information to help elucidate the impact of a hormonal cervical relaxation protocol upon luteal and endocrine function in superovulated sheep submitted to NSER.

Our findings using a PGF-OT cervical relaxation protocol in superovulated sheep is reminiscent of previous studies on ewes with synchronized estrus [13] and superovulated goats [6], demonstrating that a single d-cloprostenol treatment 16 h before NSER associated with OT is efficient to promote cervical relaxation. Here we observed similar efficiency in cervix-transposing rates and time intervals for all the steps of the procedure among the three ex-

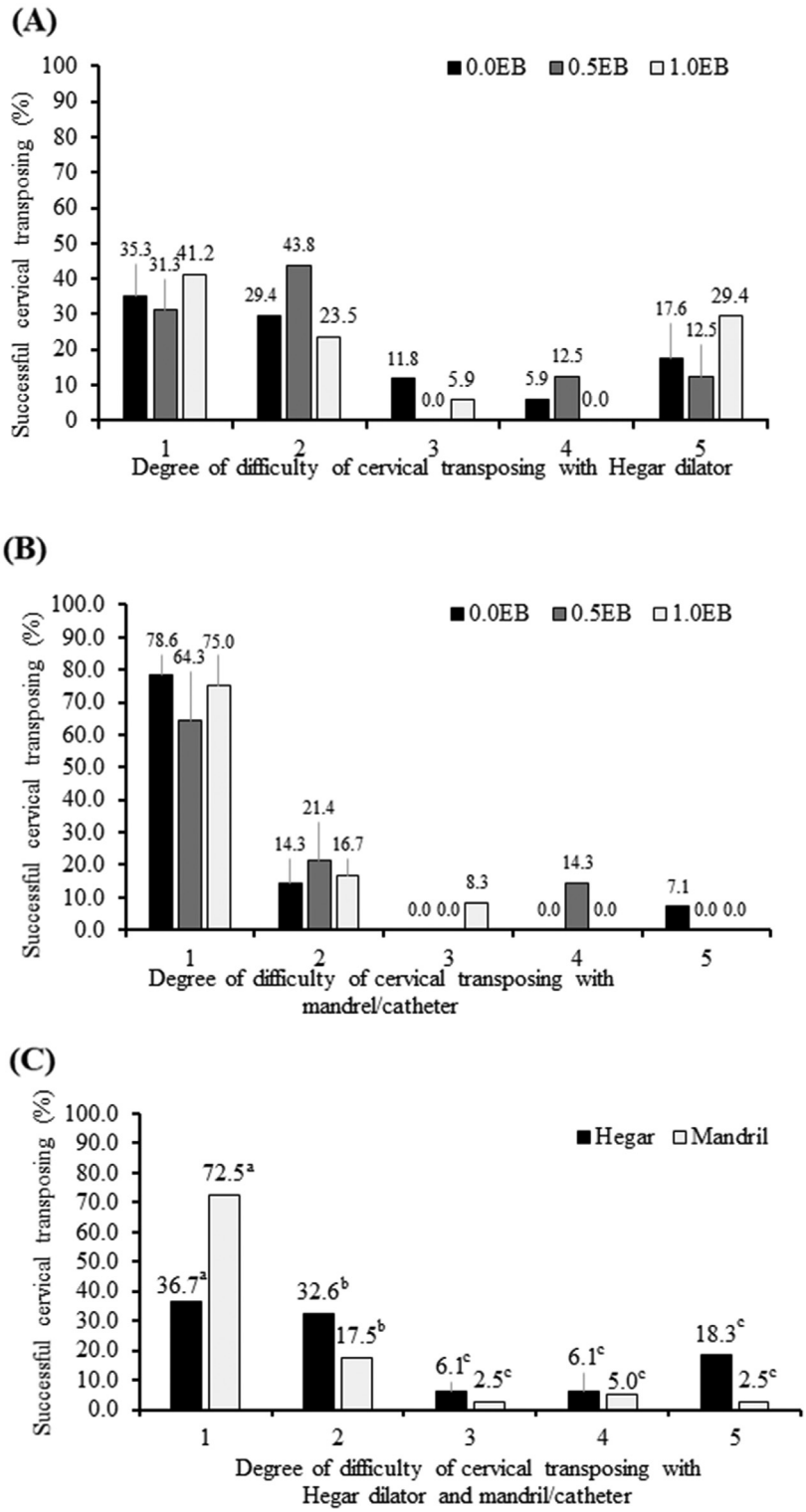

Fig. 2. Successfully cervical transposing rates (y-axis) in ewes submitted to a 9-day progesterone-based estrus synchronization protocol associated with a superovulatory treatment using 333 IU of pFSH (six decreasing doses starting 60 h before P4 device removal), and a cervical relaxation protocol using different doses of estradiol benzoate (EB; 0.0, 0.5 or 1.0 mg) associated with PGF2α (d-cloprostenol) and oxytocin before non-surgical embryo recovery (NSER). The x-axis represents the degree of difficulty in passing through transposing the cervix with the Hegar dilator (**A**), mandrel and catheter equipment (**B**) or both (**C**). Scale 1 to 5, where (1) very easy, time to pass cervix <1 min; (2) easy, time to pass cervix 1 to 3 min; (3) moderate difficulty, time to pass cervix 3 to 7 min; (4) difficult; time to pass cervix 7 and 10 min; and (5) impossible to penetrate the cervix with dilator or unable to complete cervical passage. [a,b]Letters differ between groups and procedure in the same degree of difficulty (Wilcoxon test; $P < 0.05$).

Table 3
Luteal characteristics on D16 and D17 (immediately before and after cervical relaxation protocol, respectively) in superovulated ewes submitted to a protocol with different doses (0.0, 0.5 and 1.0 mg) of estradiol benzoate (EB) plus 37.5 μg of d-cloprostenol 16 h before and 50 IU of oxytocin 20 min prior to non-surgical embryo recovery (NSER).

Endpoints	D16			D17		
	0.0EB	0.5EB	1.0EB	0.0EB	0.5EB	1.0EB
Overall CL count	10.1 ± 2.2	8.3 ± 1.1	10.8 ± 2.1	10.1 ± 2.2	8.3 ± 1.1	10.8 ± 2.1
CL diameter (mm)	81.1 ± 14.9[a]	59.5 ± 7.5[a]	62.3 ± 11.2[a]	65.8 ± 12.1[b]	54.5 ± 5.9[b]	53.9 ± 9.2[b]
CL volume (mm^3)	2635.3 ± 476.1[a]	1710.7 ± 287.4[a]	1802.4 ± 320.1[a]	1492.6 ± 303.9[b]	1232.8 ± 153.4[b]	859.1 ± 184.7[b]
CL area (mm^2)	436.7 ± 78.7[a]	309.7 ± 43.8[a]	313.0 ± 58.4[a]	301.2 ± 56.5[b]	255.5 ± 29.4[b]	243.6 ± 38.6[b]
Ovarian blood perfusion (%)	27.0 ± 2.1	30.1 ± 2.8[a]	24.1 ± 2.1	20.6 ± 2.9	21.0 ± 3.1[b]	24.5 ± 1.5
CL echogenicity	60.9 ± 3.2	62.0 ± 3.0	59.0 ± 3.3	57.6 ± 2.2	54.1 ± 1.5	60.0 ± 1.4
CL heterogeneity	9.7 ± 0.7	8.9 ± 0.5	9.9 ± 0.4	9.5 ± 0.5	9.1 ± 0.5	9.3 ± 0.4

CL, corpus luteum.
[a,b] Different letters indicate differences between the moments evaluated within the same group ($P < 0.05$). * $P > 0.05$ for comparison between group on the same day.

perimental groups, indicating the success and feasibility of this new cervical relaxation protocol for NSER in crossbred sheep. This lack of differences in time to execute the procedures among groups contradicts a previous study by our group, which reported that it took longer to pass the Hegar dilator and mandrel-catheter through the cervix in 0.0EB group compared with females receiving either 0.5 or 1.0 mg EB [13]. This apparent contradiction may be explained by the greater number of oxytocin receptors after superovulation treatments [16]. This study also confirm the efficiency of the NSER technique in nulliparous females, in disagreement with previous studies reporting that NSER was not feasible for this animal category [17,18]. Perhaps the success of NSER is more related to the individual cervical anatomy than their reproductive status or parity. Degrees of difficulty in cervical transposition regardless of the treatment used for relaxation were still observed in this study, as well as in Dias et al. (2020). This corroborates once again that 1) the cervical anatomy of the species is a major obstacle to a successful performance of the technique and 2) it is necessary to previously select the animals for the existence of visible abnormalities on visual inspection that could compromise NSER procedure efficiency [2,19].

The higher recovery rate observed in the 0.5EB group and nulliparous females may be due to a reduction in the accuracy of the ultrasound evaluation. According to Pinto *et al.* (2017) [20], animals with a high superovulatory response (>8 CL) have a decreased accuracy and sensitivity of luteal count by ultrasound compared with poor responders (≤4 CL). Still, ultrasound luteal counting remains an effective, less invasive, real-time alternative to assess the number of CL on the ovaries. The occurrence of multioocyte follicles has already been described in sheep and goat primordial follicles [21,22], but the literature has no reports of that particular phenomenon in antral follicles. Nonetheless, this may explain the lack of agreement luteal counting and recovered structures. Finally, as expected, females with a higher recovery rate has also a higher mean number of viable embryos recovered.

Previous information suggests that the mechanism by which PGF promotes cervical relaxation involves triggering luteolysis and the growth of preovulatory follicles [23]. During the early luteal phase, the pattern of prostaglandin secretion is controlled primarily by the higher concentrations of P4, which inhibits E2 and OT endometrial receptors until the end of the luteal phase. After an exogenous treatment with PGF, however, P4 levels decrease and E2 receptors are bound by circulating estrogen, stimulating OT receptors, which eventually triggers the release of more prostaglandins, culminating in luteolysis [24]. This explains why not only EB-treated groups had an increase in E2 concentration on D17, but also 0.0EB group had an almost two-fold increase in E2 concentration 16 h after treatment. In addition, we observed a decrease in P4 concentration associated with the changes in plasma E2 levels on D17, corroborating the proposed mechanism of cervical dilatation as a result of luteolysis, similar to what has demonstrated in superovulated goats [6].

Association of the P4 dosage and the evaluation of CL morphology (i.e., diameter, area, and volume) [25] and echotexture [26,27] have been used as tools to assess luteal functionality during estrous cycle. It can be noted that the cervical relaxation protocol directly interfered on the luteal function of superovulated ewes, regardless of the use of EB treatment, not only because of the changes in P4 and E2 levels, but also for a noticeable effect on CL morphology, with a reduction in luteal diameter, volume, and area on D17. The exogenous administration of PGF induced luteolysis, leading to a functional and physical regression of the CL. This was a result of the loss of communication between endothelial and steroidogenic cells, reducing the blood supply to the luteal tissue and, consequently, dropping P4 concentration [28]. However, neither effect on echogenicity nor heterogeneity was observed. According to Davies et al. [29] and Arashiro et al. [26], the correlation between steroidogenic function and echogenicity is low and poor, limiting the use of this tool as a predictor of luteal function, while heterogeneity seems to be cycle-dependent [27], which would justify the lack of any significant difference between groups and moments of observation.

It is important to verify how the exposure to an environment of unexpected hormonal changes can directly or indirectly affect the viability and post-transfer survival of embryos collected by NSER. Previous studies have shown that goat embryos collected non-surgically from donors treated only with a PGF-analogue [11,12,30,31] or sheep

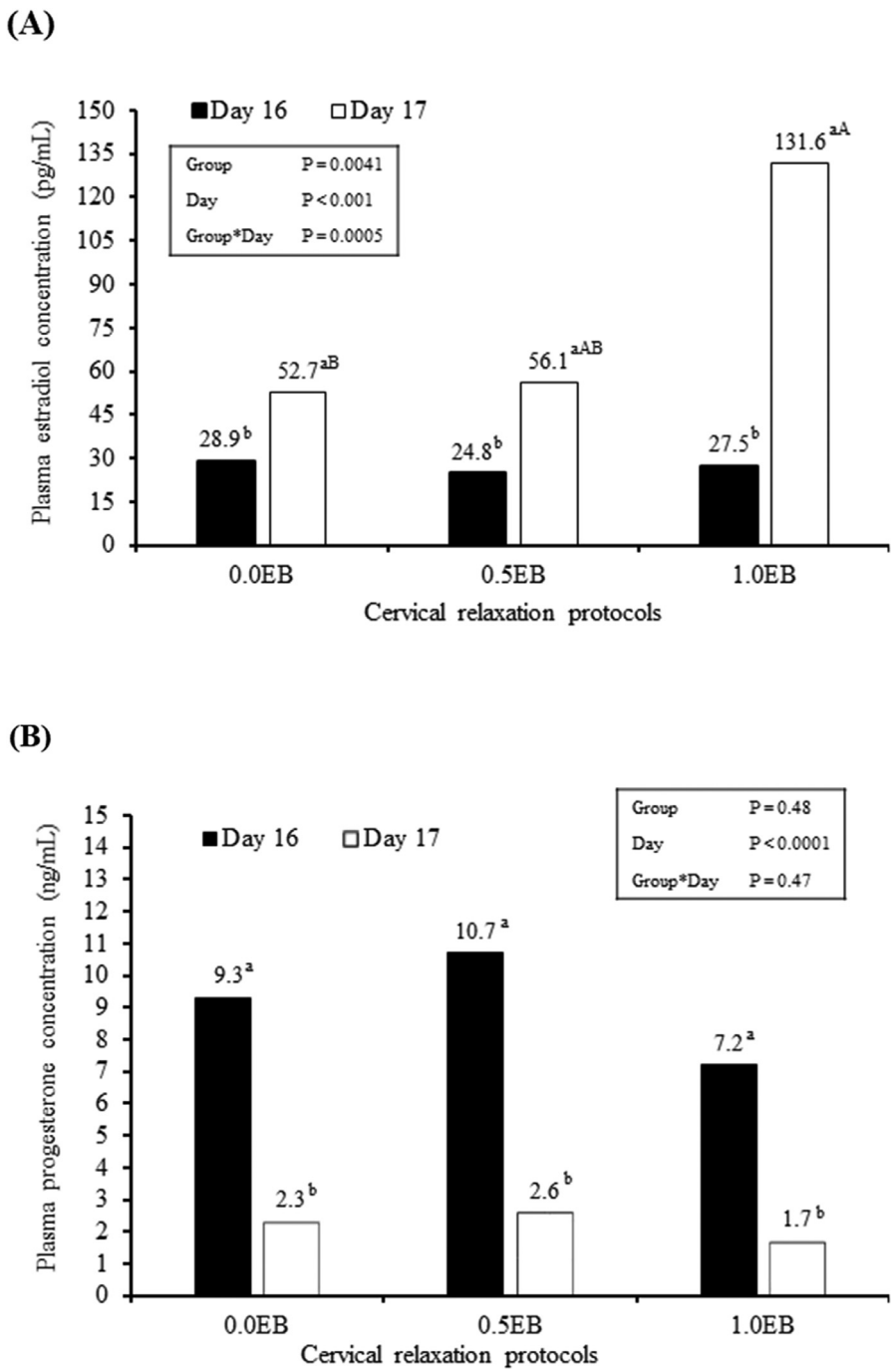

Fig. 3. Plasma concentrations of estradiol (**A**) and progesterone (**B**) on D16 and D17 (immediately before and after cervical relaxation protocol, respectively) in ewes submitted to a 9-day progesterone-based estrus synchronization protocol associated with a superovulatory treatment using 333 IU of pFSH (six decreasing doses starting 60 h before P4 device removal), and a cervical relaxation protocol using different doses of estradiol benzoate (EB; 0.0, 0.5 or 1.0 mg) associated with PGF2α (d-cloprostenol) and oxytocin before non-surgical embryo recovery (NSER). [a,b]Different lowercase letters indicate differences between days within the same group; [A,B]Uppercase letters indicate differences between groups within the same day (Tukey; $P < 0.05$).

donors [32] receiving d-cloprostenol and EB resulted in acceptable pregnancy rates after the transfer of fresh or frozen-thawed embryos. Likewise, treatment with PGF and OT prior to NSER in superovulated goats did not affect the viability of collected embryos [6]. Although progesterone levels did not differ between groups, it is noteworthy that P4 was near to basal levels in the 1.0EB group, which may affect embryonic viability to some extent, considering that adequate circulating P4 is essential for embryonic survival [33].

5. Conclusion

This study provides evidence that NSER can be efficiently performed after a cervical relaxation protocol without the use of EB in superovulated Lacaune x Santa Inês crossbred ewes, regardless of their category (nulliparous and multiparous). One must consider the isolated contribution of d-cloprostenol on the cervical relaxation protocol to change circulating P4 and E2 profiles. The feasibility of NSER in nulliparous females opens a new frontier for the use of assisted reproduction in young females, further expanding the spectrum of use of NSER in sheep.

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Declaration of Competing Interest

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

CRediT authorship contribution statement

J.H. Dias: Methodology, Validation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **J.D. Gonçalves:** Methodology, Investigation. **A.M. Arrais:** Methodology, Investigation. **J.M.G. Souza-Fabjan:** Writing – review & editing. **R. Bastos:** Investigation. **R.I.T.P. Batista:** Methodology, Investigation, Writing – review & editing. **L.G.B. Siqueira:** Writing – review & editing. **M.E.F. Oliveira:** Writing – review & editing. **J.F. Fonseca:** Methodology, Resources, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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