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ORIGINAL RESEARCH

Hormonal protocol used for cervical dilation in ewes does not affect morphological embryo quality but reduces recovery rate and temporarily alters gene expression

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Abstract

Background: Information on the impact of hormonal protocols for cervical dilation on the quality of ovine embryos is scarce.

Methods: To compare the quality of embryos after cervical dilation protocol, ewes (n = 64) were allocated into either a treated group (100 μg estradiol benzoate intravenous and 0.12 mg cloprostenol intramuscularly, 12 hours before embryo collection plus 100 iu oxytocin intravenous 15 minutes before the collection procedure) or a control group (saline). Luteal function was analysed using ultrasonography and P4 measurement. Some collected embryos were frozen/thawed for gene expression, others were cultured in vitro, frozen/thawed for gene expression, and the remaining embryos were fixed for the apoptosis test (TUNEL test).

Results: The treatment reduced fluid (p=0.04) and structure (p=0.03) recovery rates, but the morphological quality, development stage, and apoptosis incidence of the embryos were not affected by treatment. The corpora lutea of the control group had greater blood perfusion ($p = 0.002$) and greater P4 concentrations at 6, 9, and 12 h after the treatment ($p < 0.0001$). The expression of *BAX*, *BCL2*, *PRDX1*, and *HSP90* genes were not affected by the treatment. However, the embryos in the treated group had fewer *NANOG* and *OCT4* transcripts than control embryos ($p = 0.008$; $p = 0.006$, respectively). After culture, there was no difference between the groups in any gene.

Conclusion: The hormonal protocol for cervical dilation reduced the efficiency of embryo collection. In addition, the treatment induced luteolysis and a transient alteration of embryo gene expression, however there were no detectable changes in embryo morphological quality, development stage, or incidence of apoptosis.

KEYWORDS
apoptosis, corpus luteum, embryo production, progesterone, qRT-PCR, sheep

INTRODUCTION

In sheep, the morphological characteristics of the cervix (misaligned cervical rings, a long and/or tortuous cervix) determine that embryos should be collected surgically, limiting the practical application of embryo biotechnologies.[1,2] Recently, the non-surgical embryo recovery (NSER) transcervical technique has become an alternative to overcome this problem,[3–5] with fewer negative effects on animal welfare.[6]

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However, this method is not applicable in every condition, as it requires technician's training and experience, and the results vary widely according to the breed, parity, and the use of cervical dilation protocols.[7–10] The dilation of the cervix with hormonal protocols might facilitate NSER as these protocols usually mimic the hormonal pattern observed during oestrous/delivery. Briefly, during the follicular phase of the estrous cycle, and during the process that triggers delivery, there are increases in estradiol, PGF2α (prostaglandin F2α), and oxytocin secretion.[11] These changes induce a reorganization of the collagen fibres of the extracellular matrix by either altering the microstructure of the fibres[12] or stimulating the production of glycosaminoglycans,[13] processes that reduce the muscular tension, relax the smooth muscle, and, consequently, dilates the cervix.[14]

Several hormones have been tested with different doses, times, and routes of administration,[15–17] but in general, it is assumed that the association of estradiol, oxytocin, and PGF2α provides interesting results.[6,10] These hormones trigger the complex cascade of events that end in cervical dilation, allowing penetration of the cervix and uterine flushing in most ewes.[3,4,18] On the other hand, embryos are normally collected during the luteal phase, in an environment impacted by high progesterone concentrations,[19] so the administration of these hormones also modifies the milieu in which the embryos are during the last hours before embryo collection. As these hormones promote uterine motility,[6,20] and can modify the activity of the uterine glands,[21,22] the treatment might also affect the local environment during a key period of embryo growth.

Considering all this information, we hypothesised that the administration of estradiol, analogues of PGF2α, and oxytocin could affect the quality of the embryos collected in NSER protocols and that this effect might be partially explained by the regression of the corpus luteum (CL). In this study, embryos were surgically collected to determine the effects of the hormonal treatment per se, without the possible differences related to the efficiency of the collection procedure.

MATERIALS AND METHODS

Ethics, animals, and facilities

All procedures were approved by the Comitê para Uso Animal of Universidade Federal Fluminense (Protocol: 9500240418). The study was performed at the Unidade de Pesquisa Experimental em Caprinos e Ovinos (22° S, 42° W) of the same university. The animals used in this study were previously evaluated by clinical and ultrasound examination, and only ewes without reproductive abnormalities and considered healthy were included in the experiment. Due to the simultaneous recording of several variables, the study was organised in seven repetitions, with some variables evaluated only in three or four of them. Overall, 64 Santa Inês ewes were submitted to the same oestrous synchronisation protocol and superovulatory treatment,[23] followed by natural mating. Ewes were maintained in an intensive system feed with chopped grass (*Pennisetum purpureum*; 2.0 kg dry matter/day/ewe) and concentrate (17% crude protein; 0.3 kg dry matter/day/ewe), with free access to mineral salt and water.

Experimental design

This experiment was conducted with 64 Santa Inês ewes (bodyweight: 40.8 ± 5.3 kg; age: 3.3 ± 1.3 years old; mean ± SD), and due to the time required per each animal, it was organised in seven repetitions. The repetitions included 10, 10, 10, 10, 8, 8, and 8 ewes each, half of which were allocated to one of two treatments. Ewes into each repetition were allocated blocking their bodyweight and their superovulatory response (number of CLs). For this, the ovaries were observed with B-mode ultrasound 24 h before embryo collection, quantifying the number of CL. In each repetition, while half of the animals were treated with the hormonal protocol for cervical dilation, the others remained as untreated controls. The hormonal protocol for cervical dilation consisted in the administration of 100 μg estradiol benzoate intravenous (RIC-BE; Agener Union, São Paulo, Brazil), diluted in 2.5 ml absolute ethyl alcohol and 2.5 ml saline solution, and 0.12 mg cloprostenol sodium intramuscularly (Estron; Agener União, São Paulo Brazil) 12 hours before the embryo collection. In addition, 100 iu of oxytocin (Ocitocina Forte UCB; Centrovet, Goiânia, Brazil) was administered via intravenous 15 minutes before the embryo collection procedure.[10] The ewes of the control group received saline solution to replace the hormones used in the treated group with the same volumes, routes, and times. Embryo recovery was performed by laparotomy on Day 6–7 after the last mating.

All the data related to embryo yield, quality, and developmental stages were measured and recorded in all ewes. In the first four repetitions, the blastocysts (grades I and II) were recovered and frozen/thawed for the analysis of gene expression. During these repetitions, the CL blood perfusion was also evaluated, and the progesterone concentrations were measured (see Section Luteal function). The embryos produced in the last three repetitions were used to measure the apoptotic index, and the remaining embryos were cultured in vitro, and also frozen/thawed for analysis of the relative expression of the same genes (Figure 1).

Luteal function

Ultrasound observations of the ovaries were performed using a portable Doppler ultrasound equipment (Sonoscape S6; Shenzhen, China) with a 7.5 MHz linear transrectal transducer adapted for use in small ruminants. Initially, the ovaries were located and scanned using B-mode ultrasound; thereafter, the colour-Doppler mode was activated and luteal blood flow was evaluated using a subjective score scale (ranging from 1 to 4) according to the proportion of

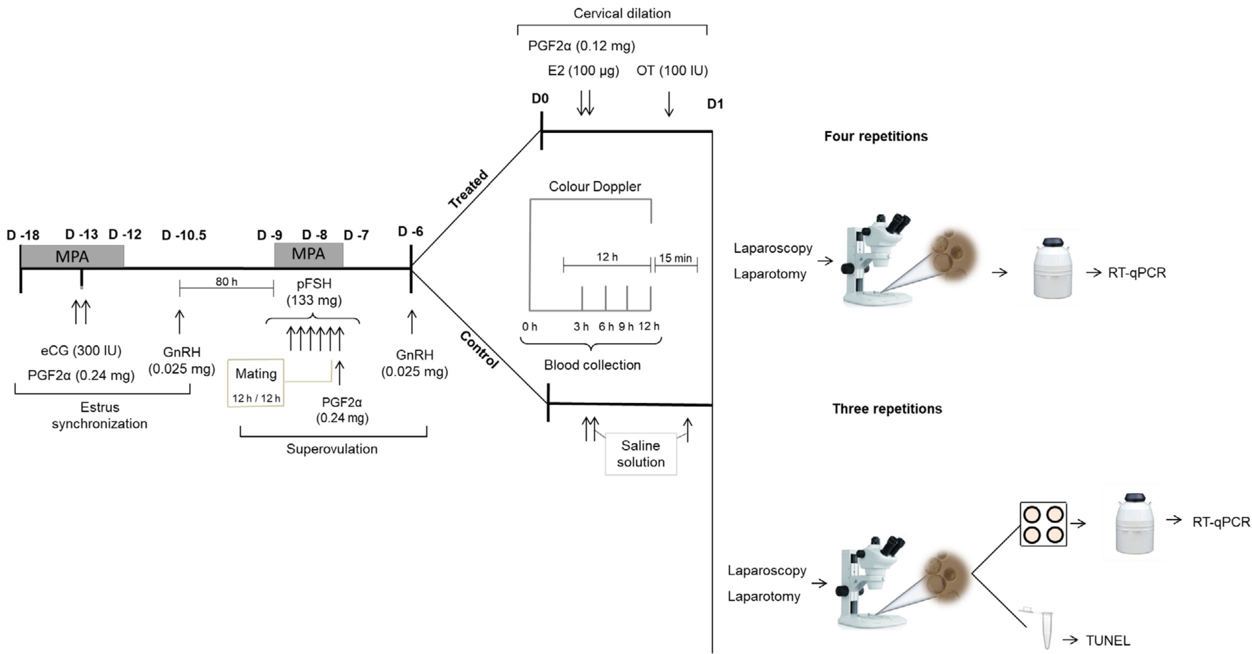

FIGURE 1 Schematic representation of the experimental design of the study. Treated: animals that received the cervical dilation protocol; control: animals that received saline solution; MPA: medroxyprogesterone acetate; PGF2α: prostaglandin F2α: cloprostenol sodium; GnRH: gonadotrophin-releasing hormone: gonadorelin acetate; E2: estradiol: estradiol benzoate; OT: oxytocin; colour Doppler: colour Doppler mode ultrasonography

coloured pixels in the luteal area, according to Arashiro and others,[24] that is, 1 (0–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). Then, the average blood perfusion of the CLs recorded in both ovaries was calculated for the statistical analysis. These evaluations were performed immediately before embryo collection (12 h after the application of the treatment).

Blood samples were collected by jugular venepuncture in the same animals, before the administration (considered time 0 h) of either the cervical dilation or saline protocol, and 3, 6, 9, and 12 hours after the application of hormones or saline. The blood samples were centrifuged at 1500 g (gravity) for 25 min, and the serum was then separated and frozen at −20°C in duplicates. Progesterone concentrations were measured by solid-phase radioimmunoassay using commercial kits (catalogue no: #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 duplicate. 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.

Surgical embryo collection and embryo evaluation

The embryo collection was performed by laparotomy (to separate the possible effects of the collection technique and due to high difficulty in transposing the cervix of ewes from the control group – saline only). All the ewes remained without access to food and water for 36 h before the procedure. Thereafter, the animals were sedated with 0.1 mg/kg acepromazine maleate (Acepran; Vetnil, Louveira, Brazil) and 0.3 mg/kg diazepam (Diazepam; Teuto, Anápolis, Brazil) both intravenous, for the laparoscopy and embryo collection. General anaesthesia was administered with induction at a maximum dose 4.0 mg/kg of propofol intravenous (Provive 1%; União Química, São Paulo, Brazil) and 6.0 mg/kg ketamine intravenous (Cetamin; Syntec, São Paulo, Brazil). The deep anaesthesia plane was maintained with 3% isoflurane (Isoforine; Cristália, São Paulo, Brazil) with the aid of inhalator anaesthesia equipment (HB Hospitalar; São Paulo, Brazil).

The laparoscopy was performed with Hopkins rigid optics (5 mm; Karl Storz Endoscopes GmbH & Co., Germany) adapted to a video system with the ewe maintained in the Trendelenburg position, as described by Bruno-Galarraga and others,[25] for confirmation of the number of CLs. After scanning the ovaries and counting the CLs, the uterus was identified with the laparoscope and a small incision was made in the midline to enable access by Babcock forceps, which exteriorised and fixed the uterus until repositioning the animal in the supine position. Then, the uterus was exposed and the embryo collection by laparotomy was performed in all the animals by the same technician according to Pinto and others.[2] The animals received an anti-inflammatory (0.5 mg/kg intramuscular; Maxican; Ourofino, São Paulo, Brazil) for three following days, and three applications of antibiotic (20 mg/kg intramuscular; Terramicina/LA; Zoetis, São Paulo, Brazil) every 48 h.

The fluid collected was observed under a stereomicroscope (20–40× magnification; Nikon, Tokyo) to

locate the structures recovered, and classified them according to their morphological quality (grades I–IV). The stages of embryo development (initial or compact morulae, initial blastocyst, blastocyst, expanded or hatched blastocyst) were evaluated as recommended by IETS.[26] Degenerated embryos, unfertilised oocytes, zona pellucida, and embryos of all grades and stages of development were considered recovered structures (treated $n = 123$; control $n = 145$). The embryos classified as grades I and II were stored in pools (five embryos from five different animals) for analysis of the transcripts involved in maintaining pluripotency (*OCT4* and *NANOG*), cell stress (*HSP90* and *PRDX1*), and apoptosis (*BCL2* and *BAX*). However, in the last three repetitions, the embryos were first cultured in vitro for 24 h and then submitted to freezing/thawing and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of the aforementioned genes. The remaining embryos were fixed immediately after collection (before in vitro culture) in 4% paraformaldehyde at 4°C to analyze the incidence of apoptosis.

In vitro embryo culture

Before the in vitro culture, the embryos (treated $n = 22$; control $n = 21$) were washed three times in holding medium (phosphate-buffered saline (PBS) supplemented with 10% fetal bovine serum [FBS]) and once in synthetic oviduct fluid (SOF) medium (BIOK SOF; Bioklone Reprodução Animal, Jaboticabal, São Paulo, Brazil), supplemented with 10% FBS. For the 24 h culture, the embryos were transferred to drops of 25 μl SOF medium plus 10% FBS under mineral oil to an incubator at 38.5°C under 5% CO_2 and saturated humidity.

RNA extraction, reverse transcription, and quantitative PCR amplification

This analysis was performed using the embryos frozen immediately after collection in the first four repetitions, as well as in the embryos collected and cultured for 24 h in the last three repetitions. Four or three pools of five grade I or II embryos (repetitions one to four, and five to seven, respectively) produced from five different animals per group were used for mRNA extraction using an RNeasy micro kit (Qiagen Inc., Valencia, USA) according to the manufacturer's instructions. Briefly, poly-A II solution (5 μl) and RLT buffer with β-mercaptoethanol (75 μl) were added to each sample for resuspension. Thereafter, 70% ethanol (75 μl) was mixed into the sample. The content was transferred to an RNeasy MinElute spin column from the RNeasy micro kit (for RNA binding to the column). DNase treatment (80 μl) was performed for 15 min at room temperature for RNA purification. After two washes, the RNA was eluted with RNA-free water (12 μl). Then, the RNA of each pool was quantified with 1 μl of the sample by spectrophotometry (Nanodrop 2000; Wilmington, DE, USA) and the samples were standardised to the same final concentration with the addition of RNAse-free water.

Reverse transcription was performed with SuperScript IV First-Strand Synthesis Supermix (Invitrogen; Carlsbad, CA, USA) with the addition of the following samples to RNA: Oligo $d(T)_{20}$ primers (2.5 μM; Promega, Madison, EUA); Superscript IV Reverse Transcriptase (200 U/μl; Invitrogen; Carlsbad, CA, USA); dNTPs (0.5 mM each; Promega; Madison, EUA); ribonuclease inhibitor (2 U/μl; Promega; Madison, USA); SSIV buffer (1×); DTT (5 mM), the sample of the extracted RNA (3.9 ng/μl) and RNA-free water to achieve a final reaction volume of 20 μl. The reverse transcription process was carried out under the following conditions: The mixture was heated to 65°C for 5 minutes, incubated on ice for at least 1 minute, and again incubated at 50°C for 10 minutes, and the reaction was inactivated at 80°C for 10 minutes. Negative or RT white controls were prepared under the same conditions without the inclusion of reverse transcriptase. Specific primers for the amplification of each gene (Table 1) were tested and their efficiency was proved through a standard curve. Relative quantification was performed in triplicate for the *BAX*, *BCL2*, *PRDX1*, *HSP90*, *NANOG*, and *OCT4* genes using a real-time polymerase chain reaction (ABI Prism 7300 Sequence Detection Systems; Foster City, CA, USA). The reactions were composed of SYBR green kit (10 μl; Power SYBR Green; Applied Biosystems, California, USA), 0.1 μM primers, nuclease-free water, and reverse-transcribed cDNA (1 μl). Template cDNAs were denatured at 95°C for 10 minutes, followed by 40 cycles of an amplification program of 95°C for 15 seconds, 55°C for 15 seconds, and 60°C for 30 seconds. Fluorescence data were acquired during the extension steps. After each PCR run, a melting curve analysis was performed for each sample to confirm that a single, specific product was generated. Relative quantification was performed by the comparative quantification method Ct ($2^{-\Delta\Delta Ct}$) using the REST 2008 software. For the normalisation of the expression results of these genes, *GAPDH* and *H2AZF* were used as reference genes.

Apoptosis test

Blastocysts on Day 7, grades I and II, produced from five different animals/treatment in the last three repetitions (from treated, $n = 10$; from control, $n = 12$) were analysed by TUNEL assay using a commercial kit (Dead End Fluorimetric TUNEL System; Promega, Madison, WI, USA). The embryos were washed three times in drops of 0.1 g/ml PBS-PVA, fixed in 4% paraformaldehyde in PBS for 25 minutes, and stored in PBS-PVA at 4°C. The TUNEL assay was initiated by permeabilising the embryos in 500 μl drops of 0.2% Triton X-100. The samples were then incubated in 50 μl drops of the TUNEL reaction mixture for 1 hour at 37°C in a dark incubator. Negative control embryos were incubated only in the staining

TABLE 1 Sequence of specific primers used for the qRT-PCR of sheep embryos

Gene symbols	Nucleotide sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	References
BAX	F: CCTGGGATCTTGAAACTCTCC TT R: CTGAGCCAGGCTGAAATCAAAA	60	566	Chakravarthi et al.[35]
BCL2	F: GCCGAGTGAGCAGGAAGAC R: GTTAGCCAGTGCTTGCTGAGA	60	214	Chakravarthi et al.[35]
PRDX1	F: CAAAGCAACAGCTGTTATGC R: GAGAATCCACAGAAGCACC	60	197	Sengodan et al.[36]
HSP90	F: GCATTCTCAGTTCATTGGCTATCC R: TTCTATCTCGGGCTTGTCATCAG	61	152	Gharibzadeh et al.[37]
NANOG	F: GATCTGCTTATTCAGGACAG R: TGCATTTGCTGGAGACTGAG	56	203	Bebbere et al.[38]
OCT4	F: GAGGAGTCCCAGGACATCAA R: CCGCAGCTTACACATGTTCT	56	204	Bebbere et al.[38]
GAPDH	F: GGGAAATCGTGCGTGACATTAAG R: TGTGTTGGCGTAAGGTCTTTG	60	273	Hogg et al.[39]
H2AZF	F: GTCGTGGCAAGCAAGGAG R: GATCTCGGCCGTTAGGTACTC	57	182	O'Connor et al.[40]

solution without the enzyme, and a positive control slide using DNase I was also prepared. The embryos were stained with Vectashield (Vector Laboratories Inc.; Burlingame, CA, USA) plus DAPI (4′,6-diamidino-2-phenylindole) and mounted on slides for evaluation by fluorescence microscopy (assessed with Hoechst 33342, Sigma-Aldrich, St. Louis, USA). The apoptotic index was calculated from the ratio of the number of cells containing apoptotic bodies (cells with DNA strand breaks, positive for TUNEL) per embryo to the total number of cells per embryo × 100.

Statistical analysis

The following variables were recorded and considered as endpoints: total recovered structures, viable embryos (grades I–III embryos), freezable embryos (grades I and II embryos), structures recovery rate (total recovered structures × 100/number of CL), flushing fluid recovery rate (flushing fluid recovered × 100/amount of medium injected), and viability rate (viable embryos X 100/total recovered structures). All these variables were analysed with mixed models including the treatment as the main effect in the model, and the repetition as a random factor. The progesterone concentration was analysed as a repeated measure using the mixed procedure with time as the repeated effect, treatment, time, and their interaction as main factors in the model, and the repetition as a random factor. The compound symmetry was included as a covariance structure. The pdiff function of statistical analysis system (SAS) was used for post hoc comparisons. These data are presented as LS means ± pooled SEM.

The area of the CL perfused and the frequency distribution of the embryos in their different quality grades and stages of development was analysed using the Mann–Whitney test. The relative quantification of the genes was performed by the comparative quantification method of Ct ($2^{-\Delta\Delta Ct}$) using the REST 2008 software.

Differences were considered as significant when $p < 0.05$.

RESULTS

Embryo recovery, embryo yield, quality, and stage of development

When comparing both experimental groups, the recovered structures, viable embryos, freezable embryos, rate of viability, and rate of freezing embryos were not affected by the treatment. The protocol for dilation of the cervix reduced the flushing fluid and structure recovery rates ($p = 0.04$ and $p = 0.03$, respectively) (Table 2). The frequency of embryos from different stages of development, quality grades, and the association of both did not differ between the groups.

Luteal function

The CL from control animals had greater blood perfusion values than the treated ones (2.5 ± 0.1 vs. 1.7 ± 0.1; $p = 0.002$) after administration of the treatment. There were effects of the treatment, time of evaluation, and an interaction between them ($p < 0.0001$, $p = 0.02$ and $p < 0.0001$, respectively) in serum P4 concentrations (Figure 2).

Gene expression and apoptosis in embryos

The treatment decreased the mRNA amount of *NANOG* and *OCT4* genes in the embryos analysed ($p = 0.008$ and $= 0.006$, respectively). The treatment did not affect the relative expression of the transcripts

TABLE 2 Summary responses (mean ± SEM or per cent) of ewes submitted to cervical dilation and the control group*

End points	Groups: Treated	Control	*p*-value
No. of ewes	32	30	
No. of CL	6.9 ± 0.9	6.8 ± 0.9	ns
No. of recovered structures per ewe (total)	3.7 ± 0.6 (123 ± 0.7)	4.8 ± 0.6 (145 ± 0.5)	ns
No. of viable embryos per ewe (total)	2.4 ± 0.6 (76 ± 0.6)	3.3 ± 0.6 (97 ± 0.5)	ns
No. of freezable embryos per ewe (total)	2.3 ± 0.6 (72 ± 0.6)	2.9 ± 1.0 (87 ± 0.4)	ns
Fluid recovery efficiency (%)	91.7 ± 2.0 [b]	96.5 ± 0.8 [a]	0.04
Recovery of structures (%)	58.2 ± 7.0[b]	75.9 ± 6.7[a]	0.03
Viability rate (%)	57.4 ± 9.4	69.0 ± 9.1	ns
Rate of freezing embryos (%)	47.6 ± 9.6	61.2 ± 9.6	ns

Note: Different letters, on the same line, are statistically distinct ($p < 0.05$).
Abbreviations: CL, corpus luteum; ns, not significant
*Protocol: 100 μg estradiol benzoate (intravenous) and 0.12 mg cloprostenol sodium (intramuscular) were administered 12 hours, and 100 iu oxytocin (intravenous) was given 15 minutes before the embryo collection procedure.

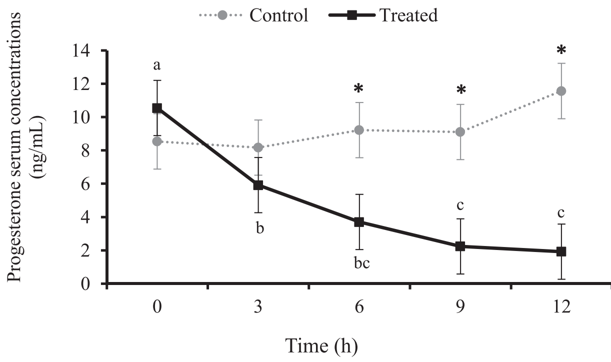

FIGURE 2 Serum progesterone concentrations (ng/ml) in ewes submitted (treated) or not (control) to the hormonal protocol for cervical dilation at different moments: 0 hour (before of the treatment – cervical dilation or control) and 3, 6, 9 and 12 hours after administration of the protocols (treated or control). Means with an asterisk (*) differ between the experimental groups; different letters (a, b, c) indicate differences in each experimental group (treated or control) ($p < 0.05$). Means followed by the same letter (or no letter) are not significant

associated with apoptosis (*BAX* and *BCL2*) and cell stress (*PRDX1* and *HSP90*). There were no differences in the analysed genes of frozen/thawed embryos after 24 hours of in vitro culture (Figure 3). There was no effect ($p > 0.05$) of the treatment on the incidence of apoptosis in the embryos (treated: 3.8 ± 3.4% [104 ± 28 cells/embryo] vs. control: 2.0 ± 2.5% [96 ± 22 cells/embryo]).

DISCUSSION

The results demonstrate that although the cervix is effectively relaxed, the hormonal treatment also induced luteolysis, which promotes important changes in the milieu in which embryos remain during the time elapsed from the treatment to the collection. In effect, CL blood perfusion decreased, and progesterone concentrations, withdrawn very quickly to basal levels; therefore, decrease of progesterone associated with the hormones administered (estradiol, oxytocin, and PGF2α), probably increased the motility of the reproductive tract, triggering a faster advance of the embryos as well as changes in the composition of the local fluids.[27,28] In a previous study, this hormonal combination modified the uterine tone, ending in a lower effectiveness of the surgical procedure.[6] As all collections were done surgically in the present study, it was possible to note that the hormonal treatment affected also the flushing effectiveness, as flushing fluid and structures recovery rates were lower in this group. Although it could not be confirmed with this experimental approach, it is possible that the changes in the tone of the tract muscles, as well as the fluid composition, partially explain the lower recovery achieved after this treatment. Overall, although hormonal treatments allow relaxing the cervix and, thus, avoiding the need of surgical procedures,[5,18] this study highlights that the cervical dilation protocol may affect the recovery rate. In addition, embryo losses through the cervix/vagina induced by the treatment for cervical dilation should not be discarded.

It is worth mentioning that these effects were detected in the recovery rates, but not in the number of embryos from the different qualities, including freezable embryos. Likewise, Wulster-Radcliffe et al.[29] observed that treatment with estradiol and oxytocin in recipient ewes did not affect the development and quality of the embryos. However, those authors were unable to distinguish whether this finding was due to the action of hormones or to the reduction of tissue damage (cervical and/or uterine) as a result of the cervical dilation and shorter manipulation times in the ewes. However, it should be considered that the time from the beginning of luteolysis to embryo collection would have been too short to impact embryo macroscopic morphology, although it might have affected at the cell or molecular levels. On the other hand, at least in the variables studied, this modification of the embryo milieu might have affected only transiently the expression of the *OCT4* and *NANOG* genes, and its levels were recomposed after culture in vitro. The authors can also not discard that it would be

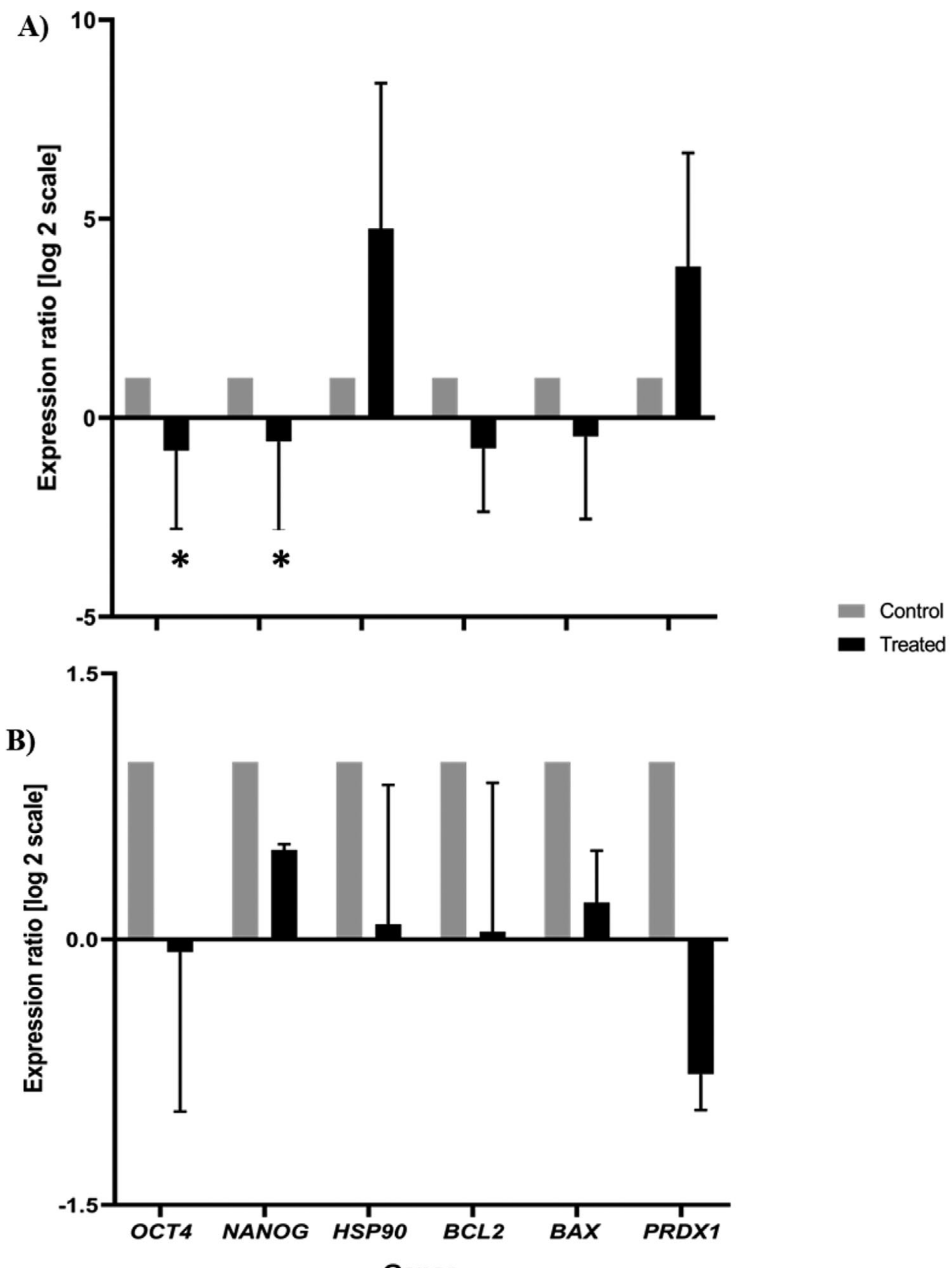

FIGURE 3 Relative gene expression (*OCT4, NANOG, HSP90, BCL2, BAX* and *PRDX1*) in ovine embryos (blastocysts grades I and II) obtained from Santa Inês ewes submitted to either the hormonal protocol for cervical dilation or a control treatment (saline solution). Embryos were either (a) immediately frozen after embryo collection (n = four pools of five grade I or II embryos per group) or (b) collected and in vitro cultured (n = three pools of five grade I or II embryos per group) for 24 hours in Synthetic Oviduct Fluid (SOF) medium in 5% CO_2 at 38.5°C and then frozen. The differences in mRNA expression of embryos from the treated group (cervical dilation protocol) and the control group were shown after normalisation to the reference genes (*GAPDH* and *H2AFZ*). The mRNA abundance in embryos from the control group was arbitrarily set to onefold. *A statistical difference between experimental groups ($p < 0.05$)

necessary to increase the number of embryos to detect these effects, but in that case, it would have a limited impact on biological consequences. In addition, the effect on embryo development beyond 12 hours of treatment and pregnancy establishment should be further investigated.

Despite the absence of macroscopic harm, the embryo's molecular machinery appears to be temporarily compromised. Thus, an alternative to alleviate these effects could be the reduction of the period between hormonal application and NSER, and thus, a shorter exposure to the luteolytic process. It has also been considered that cervical dilation can be achieved with oxytocin alone,[30–32] but its use is still controversial. Although it allows good rates of cervical penetration, there are reports of lower pregnancy and lambing rates.[20,30] Therefore, although the combination of estradiol, oxytocin, and PGF2α seems to be an effective alternative for cervical dilation, and resulted in reasonable pregnancy and lambing rates after the transfer of frozen-thawed ovine embryos,[33] it would be important to study the possibility of shortening the period in which these hormones are acting, and/or decreasing their doses. This may also

differ according to each breed, as variability in the cervix morphology[34] could limit the access with the same treatment.

CONCLUSION

The hormonal protocol for cervical dilation led to lower recovery rate, induced luteolysis, and a transient alteration of embryo gene expression. However, there were no detectable changes up to 12 hours after treatment in morphological embryo quality, the development stage, or the incidence of apoptosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS APPROVAL

The procedures performed in this study were analysed and approved by the Comitê para Uso Animal of Universidade Federal Fluminense (Protocol no.: 9500240418).

AUTHOR CONTRIBUTIONS

Juliana D. R. Santos: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization; Ribrio I. T. P. Batista: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing; Visualization; Rodolfo Ungerfeld: Validation, Formal analysis, Writing – original draft, Writing – review & editing; Augusto R. Taira: Methodology, Investigation; Caroline G. Espírito Santo: Methodology, Investigation; Joanna M. G. Souza-Fabjan: Methodology, Validation, Writing – original draft, Writing – review & editing; Visualization; Daniel A. M. Fernandes: Methodology, Investigation; Mário F. A. Balaro: Methodology, Validation, Investigation; Isabel O. Cosentino: Methodology, Investigation; Viviane L. Brair: Methodology, Investigation; Pedro H. N. Pinto: Methodology, Investigation; Ana Beatriz S. Carvalho: Methodology, Investigation; Jeferson F. Fonseca: Methodology, Investigation, Writing – review & editing; Felipe Z. Brandão: Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

PEER REVIEW

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