Theriogenology 218 (2024) 208–213

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

The presence of estradiol benzoate in the cervical relaxation treatment for non-surgical embryo collection does not impair embryonic morphological quality, cryosurvival, and gene expression profile

Ribrio Ivan Tavares Pereira Batista [a,*], Jenniffer Hauschildt Dias [b], Joedson Dantas Gonçalves [c], Gabriel Brun Vergani [c], Maria Emilia Franco Oliveira [c], Joanna Maria Gonçalves Souza-Fabjan [a], Jeferson Ferreira Fonseca [d]

[a] Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, 24230-340, Niterói, RJ, Brazil
[b] Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n, 36570-000, Viçosa, MG, Brazil
[c] Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de acesso Prof. Paulo Donato Castellane, s/n, 14884-900, Jaboticabal, SP, Brazil
[d] Embrapa Caprinos e Ovinos, Núcleo Regional Sudeste, Rodovia MG 133, Km 42, CEP 36155 - 000, Coronel Pacheco, MG, Brazil

ARTICLE INFO

Keywords:
Cervical dilation
Embryo
Cryopreservation
Gene expression
NSER
Ovine

ABSTRACT

Non-surgical embryo recovery (NSER) is usually preceded by a cervical relaxation in ovine donors, based on estradiol benzoate (EB), prostaglandin (PGF), and oxytocin (OT). However, it is hypothesized that, due to poorly understood mechanisms, EB can result in embryotoxic actions. To evaluate this, 20 min before NSER superovulated sheep were induced to cervical relaxation with 0.0 (G0.0), 0.5 (G0.5), or 1.0 mg (G1.0) of EB associated with 37.5 μg of PGF 16 h before NSER and 50 IU of OT. In doing so, the efficiency and duration of the NSER procedure showed no compromise ($P > 0.05$). Additionally, the presence of EB did not affect ($P > 0.05$) the embryo's morphological quality, the development dynamics, or the abundance of transcripts associated with embryonic quality (*OCT4* and *NANOG*), cellular stress (*HSP90* and *PRDX1*), and apoptosis (*BCL2* and *BAX*). A similar result ($P > 0.05$) was also observed when comparing embryonic cryosurvival at 24 (52.0, 52.0, and 54.0) and 48 h (60.0, 54.0, and 58.0) of *in vitro* culture (G0.0, G0.5, and G1.0, respectively). Thus, we can conclude that EB use does not compromise embryonic quality and cryoresistance.

1. Introduction

Non-surgical embryo recovery (NSER) is an effective non-invasive technique for embryo recovery procedures in small ruminants. In different sheep breeds, the technique achieves a successful rate ranging from 70 to 95 % [1–3]. This represents an important alternative to surgical embryo collection methods (laparotomy and laparoscopy), which may prove crucial for a consumer market increasingly worried about animal welfare in the production chain. Furthermore, the use of this technique allows for the reduction of intervals between collections and anatomical changes – such as adhesions – which surgical embryo collection requires. Notably, the success of the NSER procedure depends on the previous induction of cervical relaxation, which is usually based on prostaglandin F2α (PGF), estradiol benzoate (EB), and oxytocin (OT) administrations [4]. These hormones relate to extracervical matrix remodeling, inducing the dispersion of collagen fibers and increasing the cervix flexibility [5]. The major problem, however, is that these are also associated with the luteolytic process, making them undesirable in superovulated ewes [6].

Physiologically, the cervical relaxation protocol may trigger deleterious effects that are similar to the early luteal regression and may be harmful to the embryo. A PGF discharge, induced by OT and/or E2 during the early luteal phase, plays an embryotoxic role in reducing fertility in estrus-induced ewes [7] and impairs embryo quality and recovery rates in superovulated ewes [8]. Endocrinologically, cervical relaxation protocol administered six days after ovulation has been shown to affect luteal function by reducing luteal area and progesterone concentration [6,9], at the same time as increasing estradiol levels by approximately two to five-fold [6]. To date, the embryo-level effects of administering these luteolytic drugs before embryo recovery have not been fully elucidated.

It is well known that, after cervical relaxation protocols with PGF

* Corresponding author.
E-mail address: ribrio@yahoo.com.br (R.I.T.P. Batista).

https://doi.org/10.1016/j.theriogenology.2024.01.038
Received 20 October 2023; Received in revised form 8 January 2024; Accepted 28 January 2024
Available online 4 February 2024

alone in goats [10] or PGF-OT-E2 in sheep [11], fresh or frozen-thawed embryos from donors undergoing NSER achieve acceptable to good pregnancy rates. However, in addition to the recovery rate, Santos et al. [12] reported that *NANOG* and *OCT-4* (important markers of embryonic pluripotency at the blastocyst stage) had affected expression, after the cervical relaxation protocol, although these were normalized after 24 h of *in vitro* embryonic culture. Despite this observation, the authors did not observe effects on embryonic morphology or apoptotic index. In view of this, we can speculate that the time taken between the induction of luteolysis and embryonic recovery may be short enough to compromise the embryo's morphological viability. However, it is possible that changes – even if transient – at molecular or cellular levels may lead to irreversible damage to the subsequent embryo development.

Considering the endocrine impacts of cervical relaxation protocols with or without EB and its possible embryotoxic effects, this study has focused on evaluating the efficiency of NSER procedure, embryonic morphology, and *in vitro* viability and survival of cryopreserved ovine embryos recovered after cervical relaxation protocols using 0.0 mg, 0.5 mg, or 1.0 mg of EB associated with PGF and OT. Additionally, the genes involved in maintaining pluripotency (OCT4 and NANOG), cell stress (HSP90 and PRDX1), and apoptosis (BCL2 and BAX) have also been assessed.

2. Materials and methods

2.1. Ethics, location, and experimental animals

This study is approved by the Embrapa Dairy Cattle Ethics in the Use of Animals Committee (Juiz de Fora, Brazil, protocol number 6365271119). The experiment took place during the non-breeding season at Embrapa's Experimental Farm 'José Henrique Bruschi,' which is located in Coronel Pacheco, MG, Brazil (latitude 45′51″S, longitude 43°20′59″W). All animals were kept in a confinement system and provided with feed supplemented with corn silage, concentrate, and mineral salt mixture, with water available *ad libitum*. A total of 60 crossbred ewes (Lacaune X Santa Inês) aged 3.1 ± 0.2 years old, with body condition scores of 2.8 ± 0.1 (scale 1 to 5; 1 = emaciated and 5 = obese) underwent an ovulation-induced protocol receiving 0.36 g of progesterone device (D0; PRIMER PR® Caprinos e Ovinos, Tecnopec – Agener União, Taboão da Serra, Brazil) for nine days and a superovulation treatment consisting of six decreasing doses (25, 25, 15, 15, and 10 %) of 333 IU of pFSH (PLUSET®, Biogénesis Bagó, Curitiba, Brazil), beginning 60 h before P4 device withdrawal, plus two doses of 37.5 μg of d-cloprostenol (Prolise®, Agener União, Taboão da Serra, Brazil) injected i.m. concurrently with the fifth and sixth pFSH doses. After P4 device withdrawal, the ewes were observed for estrus behavior and naturally mated with fertile rams (1:7 ram to ewe ratio) every 12 h while in standing estrus. To evaluate the effect of estradiol benzoate in the cervical relaxation protocol, the sheep were divided into three groups: i) 0.0 mg (G0.0), ii) 0.5 mg (G0.5), and iii) 1.0 mg (G1.0) of estradiol benzoate. After collection, the embryos at the blastocyst stage were dry frozen in liquid nitrogen for gene expression analysis, while the rest were subjected to slow freezing and thawing and cultured *in vitro* for 48 h.

2.2. Cervical relaxation protocols, NSER procedure, and embryo evaluation

The cervical relaxation protocol started 16 h before the NSER procedure (D16). Ewes were randomly divided to receive 37.5 μg of d-cloprostenol associated with 0.0 mg (G0.0), 0.5 mg (G0.5), or 1.0 mg (G1.0) of estradiol benzoate (RIC-BE®, Agener União, São Paulo, Brazil) via i.m. An application of 50 IU of oxytocin (Ocitocina Forte UCB®, UCBVet, Jaboticabal, Brazil) was performed in all animals 20 min before the NSER procedure, with the procedures performed as described by Dias et al. (2020). Degenerated embryos, non-fertilized ova, zona pellucida, and embryos of all development grades and stages were considered recovered structures, then classified according to the International Embryo Technology Society (IETS) guidelines manual [13]. Only embryos at the compact morula and blastocysts (initial, expanded, and hatched) stages were considered viable structures and classified as grades I, II, or III.

2.3. Cryopreservation of embryos

Slow freezing procedures were performed according to Fonseca et al. [14]. The ethylene glycol (EG; 1.5 M) was used in the first step with a base solution containing PBS supplemented with 20 % fetal bovine serum. Freezing was performed by cooling from 20 °C until −6 °C at a rate of 3 °C/min; stabilization at −6 °C for 15 min and seeding after 5 min; cooling to −32 °C at a rate of −0.5 °C/min, then holding for 10 min at −32 °C, before plunging into liquid N_2 for storage. Thawing was performed at room temperature for 5 s followed by a water bath at 37 °C for 30 s.

2.4. In vitro embryo culture

Embryos (G0.0, n = 25; G0.5, n = 24; G1.0, n = 24) were washed three times in a holding medium (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). Afterward, they were *in vitro* cultured in drops containing a ratio of 2.5 μL of SOFaa medium per embryo (BIOK SOF, Bioklone Reprodução Animal, Passo Fundo, RS, BR) covered with mineral oil at 38.5 °C with 5 % CO_2 and 5 % O_2 during 48 h in a benchtop incubator (EVE, WTA, Cravinhos, SP, BR). By analyzing the rate of re-expansion at 24 and 48 h after *in vitro* culture had begun, embryo survival was evaluated.

2.5. Gene expression

For gene expression analysis, we utilized 45 blastocysts (n = 15 blastocysts per group). Immediately after collection, the pool was washed three times in PBS supplemented with 0.1 % polyvinyl alcohol and frozen in liquid nitrogen using DNase- and RNase-free cryotubes (Corning, New York, USA). Once this stage was complete, an analysis of the transcripts (Table 1) involved in maintaining pluripotency (OCT4 and NANOG), cell stress (HSP90 and PRDX1), and apoptosis (BCL2 and BAX) could take place.

2.5.1. RNA extraction

Total RNA was extracted using the RNeasyMicro Kit (Qiagen Inc., Valencia, USA), according to the manufacturer's instructions. Elution was performed with 14 μL of RNase-free water and the RNA quantification of each pool was performed using 1 μL of the sample (mean: G0.0 = 6.3 ng/μL; G0.5 = 6.8 ng/μL, and G1.0 = 7.6 ng/μL) on a spectrophotometer (Nanodrop Lite, Thermo Fisher Scientific, Wilmington, DE, USA).

2.5.2. Reverse transcription

For reverse transcription, the SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), was used for all samples in the same RNA concentration. The reverse transcription reaction was performed in a two-step mix: (1) oligo (dT) 20 primers, dNTP mix, nuclease-free water, and RNA template (mix volume: 13 μL per sample); (2) Superscript IV Reverse Transcriptase, RNaseOUT Recombinant RNase inhibitor, DTT, Superscript IV RT buffer (mix volume: 7 μL per sample). With the two-step mix complete, a final volume of 20 μL was totalized. Each first mix sample was then incubated at 65 °C for 5 min, followed by 4 °C for 3 min when the second mix was added. Afterward, the cDNA synthesis was followed by 50 °C for 10 min, 80 °C for 10 min and, finally, 4 °C for 1 min, before the samples were chilled on ice or stored at −20 °C

Table 1
Sequence of specific primers used for the RT-qPCR analysis.

Gene symbols	Nucleotide sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	References
BAX	F: CCTGGGATCTTGAAACTCTCC TT R: CTGAGCCAGGCTGAAATCAAAA	60	566	[24]
BCL2	F: GCCGAGTGAGCAGGAAGAC R: GTTAGCCAGTGCTTGCTGAGA	60	214	[24]
PRDX1	F: CAAAGCAACAGCTGTTATGC R: GAGAATCCACAGAAGCACC	60	197	[25]
HSP90	F: GCATTCTCAGTTCATTGGCTATCC R: TTCTATCTCGGGCTTGTCATCAG	61	152	[26]
NANOG	F: GATCTGCTTATTCAGGACAG R: TGCATTTGCTGGAGACTGAG	56	203	[27]
OCT4	F: GAGGAGTCCCAGGACATCAA R: CCGCAGCTTACACATGTTCT	56	204	[27]
GAPDH	F: GGGAAATCGTGCGTGACATTAAG R: TGTGTTGGCGTAAGGTCTTTG	60	273	[28]
H2AFZ	F: GTCGTGGCAAGCAAGGAG R: GATCTCGGCCGTTAGGTACTC	57	182	[29]

until quantitative polymerase chain reaction (qPCR).

2.5.3. qPCR amplification and analysis

Relative quantification was performed in triplicates using qPCR (Applied Biosystems QuantStudio 3, Thermo Fisher Scientific, Wilmington, DE, USA). Reactions (20 μL of total volume) were prepared using a mixture of SYBR green kit (10 μL; Power SYBR Green, Applied Biosystems), 0.1 μM primers (Table 1), nuclease-free water, and reverse transcribed cDNA (0.5 μL). Negative controls, comprising the PCR reaction mixture without nucleic acids, were also run with each group of samples. Template cDNA was denatured at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Fluorescence data were acquired during the extension steps. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was generated. Primer efficiency was calculated using LinRegPCR software [15] for each reaction, with the efficiency averages calculated as: 0.94 to BAX; 0.95 to BCL-2; 0.98 to PDRX1; 0.99 to HSP90; 1.04 to NANOG; 0.94 to OCT-4; 0.96 to GAPDH; and 0.96 to H2AFZ. Using the REST 2008 software [16], relative quantification was performed according to the comparative Ct method $^{(2-\Delta\Delta Ct)}$. Then, using the geometric mean of GAPDH and H2AFZ values, the expression of each target gene was normalized. Finally, the stability of the reference gene was calculated according to the methodology described by Ref. [17], using the BestKeeper – Excel tool.

2.6. Statistical analyses

The following variables were recorded and considered as endpoints: estrus response; interval to estrus; superovulatory response (%; ewes with more than 3 CL/ewes in estrus); overall CL count; overall luteinized unovulatory follicle (LUF) count; ewes with LUF (%); successful Hegar dilator transposing (%); duration of transpositions with Hegar dilator and mandrel-catheter (min); duration of uterine flushing and total duration of NSER procedure (min); successful NSER procedure (%; considering ewes successfully flushed); flushing efficiency (%); CL count in ewes successfully flushed; recovery rate (number of structures recovered x 100/total of CL count); number of ova/embryos recovered; number of transferable, freezable and degenerated embryos; number of unfertilized ova; number of zona pelucidae; viability rate (number of viable embryos x 100/total number of recovered structures); and re-expanded rates at 24 h and 48 h (number of re-expanded embryos x 100/total number of *in vitro* cultivated embryos). Statistical analyses were performed using SAS® Studio software. The Levene test was then utilized to assess the homogeneity of variances, while the error normality was verified for each variable according to the Shapiro–Wilk test. Parametric data (i.e. interval to estrus, overall CL count, overall LUF count, durations of procedures, and numbers of structures recovered) were analyzed by one-way ANOVA (PROC GLM) followed by a posthoc Tukey test to determine mean differences. Incidence rate comparison data (estrus and SOV response, successful procedures, viability rate, and re-expanded rates) were analyzed using the Chi-square test to determine statistical differences. Finally, the comparative quantification method of Ct ($2 - \Delta\Delta Ct$) using the REST 2008 software was utilized to perform relative gene quantification. Differences were considered significant when $P < 0.05$.

3. Results

3.1. NSER procedure, superovulatory response, and embryo yield

Table 2 shows the results of estrus, superovulation, NSER procedure, and embryo production. A total of 38.0 % (19/60) of animals were excluded from the NSER procedures for not presenting estrus behavior and/or SOV responses (CL count ≥3). Regardless of the EB treatment, successfully performing NSER and uterine flushing in 80.4 % (33/41) of the responding animals was possible. In terms of the duration of cervical transpositions and uterine flushing, the groups did not differ ($P > 0.05$), which was also the case in CL and/or structures recovered. The only difference was found in the recovery rate, where G0.5 had a greater average rate (115.9 ± 17.3) compared to the G0.0 group (61.2 ± 10.3; $P < 0.05$).

3.2. Embryo survival

Table 2 describes data related to embryo survival. After cryopreservation, no difference ($P > 0.05$) was found among groups for embryo survival (Table 3). However, when data are plotted independently of the experimental group, embryo survival average at 48 h of *in vitro* culture was 57 %.

3.3. Gene expression

Data related to gene expression is shown in Fig. 1. The *BAX, BCL2, PRDX1, HSP90, NANOG*, and *OCT4* genes did not differ ($P > 0.05$) between G0.0 and G0.5, or between G0.0 and G1.0 groups. The *BCL2* gene was downregulated ($P = 0.04$) in G1.0 compared to G0.5, while the other genes were similar ($P > 0.05$) between these groups.

4. Discussion

The present study data corroborates previous research [18] showing that estradiol removal from cervical relaxation protocol does not compromise the efficiency or time of NSER procedure in Lacaune x Santa

Table 2
Estrus, superovulatory, and embryo recovery endpoints (% or mean ± SEM) in superovulated[a] ewes submitted to a cervix relaxation protocol with different doses (0.0, 0.5, and 1.0 mg) of estradiol benzoate plus 37.5 μg of d-cloprostenol 16 h and 50 IU of oxytocin 20 min, all before the non-surgical embryo recovery (NSER).

	Estradiol benzoate			
Endpoints	0.0 mg	0.5 mg	1.0 mg	*P value*
Animals	23	16	21	–
Estrus response (%)	78.3 (18/23)	93.7 (15/16)	80.9 (17/21)	0.41
Interval to estrus (h)	27.3 ± 2.7	25.6 ± 2.6	26.1 ± 2.3	0.98
SOV response[c] (%)	83.3 (15/18)	73.3 (11/15)	88.2 (15/17)	0.54
Overall CL count	11.1 ± 1.3 [166]	9.2 ± 0.8 [101]	11.3 ± 1.6 [169]	0.52
Overall LUF count	1.9 ± 0.4 [29]	1.5 ± 0.4 [17]	1.5 ± 0.5 [23]	0.75
Ewes with LUF (%)	77.8 (14/18)	53.3 (8/15)	58.8 (10/17)	0.30
Successful Hegar dilator transposing rate (%)	93.3 (14/15)	81.8 (9/11)	80.0 (12/15)	0.54
Duration of Hegar dilator transposing (min)	3.7 ± 0.8	4.4 ± 1.3	3.1 ± 0.9	0.69
Duration of Mandrel-Catheter transposing (min)	2.0 ± 0.9	2.5 ± 1.1	1.7 ± 0.5	0.82
Duration of uterine flushing (min)	16.5 ± 0.8	20.5 ± 4.2	15.4 ± 0.7	0.25
Duration of NSER procedure (min)	21.3 ± 0.8	26.4 ± 3.6	22.4 ± 1.9	0.25
Successful NSER procedure rate (%)	80.0 (12/15)	81.8 (9/11)	80.0 (12/15)	0.99
Flushing efficiency (%)	100.0	100.0	100.0	–
CL count in ewes successfully flushed	11.1 ± 1.3 [133]	9.0 ± 0.9 [81]	11.7 ± 1.9 [141]	0.44
Recovery rate (%)	61.2[b] ± 10.3	115.9[a] ± 17.3	78.0[ab] ± 15.4	0.02
Ova/embryos recovered	7.1 ± 1.6 [81]	9.7 ± 1.5 [87]	9.8 ± 2.4 [118]	0.60
Transferable embryos[d]	5.3 ± 1.2 [58]	6.9 ± 1.8 [62]	6.0 ± 2.1 [72]	0.82
Freezeable embryos[d]	4.9 ± 1.0 [54]	6.9 ± 1.8 [62]	5.4 ± 1.9 [65]	0.70
Non-viable embryos	2.1 ± 1.0 [23]	2.8 ± 1.3 [25]	3.6 ± 1.3 [43]	0.68
Unfertilized ova	1.5 ± 0.8 [17]	1.0 ± 1.0 [9]	3.0 ± 1.4 [36]	0.45
Morulae	3.4 ± 0.7 [37]	2.1 ± 0.6 [19]	2.7 ± 1.5 [32]	0.75
Blastocyst	1.9 ± 0.7 [21]	4.9 ± 1.3 [44]	3.4 ± 1.5 [41]	0.28
Degenerated embryos	0.0 ± 0.0 [0]	0.0 ± 0.0 [0]	0.3 ± 0.2 [4]	0.09
Zona pelucidae	0.2 ± 0.1 [2]	0.3 ± 0.2 [3]	0.3 ± 0.2 [4]	0.79
Viability rate (%)	71.6 (58/81)	71.3 (62/87)	61.0 (72/118)	0.18

CL = corpora lutea; LUF = luteinized unovulated follicle; SOV = superovulatory.
(n/n) Number of animals, ovarian structures, or recovered ova/embryos.
[n] Total sum of animals, ovarian structures, or recovered ova/embryos.
[a,b] Within a row, means without a common superscript differed ($P < 0.05$).
[a] 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 37.5 μg d-cloprostenol at the time of the last two pFSH doses.
[c] Superovulatory response, considering ewes that manifested estrus behavior with ≥3 CL counted in D16.
[d] Transferable embryos = embryos Code 1 and Code 2; Degenerated embryos = embryos Code 4.

Ines crossbred ewes. The removal of this anabolic agent from the cervical relaxation protocol, therefore, produces an NSER collection technique that is even more aligned with market demands, taking onboard animal welfare concerns and the reduction or removal of potentially carcinogenic hormones. Nevertheless, it remains important to highlight that the use of estradiol in the cervical relaxation protocol did not affect embryonic quality. Indeed, no difference was observed in the average number of transferable embryos, freezable embryos, and non-viable embryos when the groups (G0, G0.5, and G1.0) were compared.

Table 3
Embryo survival after 24 and 48 h of *in vitro* culture of frozen-thawed embryos recovered by non-surgical embryo recovery (NSER) technique after cervical relaxation protocol using 0.0 mg (G0.0), 0.5 mg (G0.5) or 1.0 mg (G1.0) of estradiol benzoate and 37.5 μg of d-cloprostenol 16 h and 50 IU of oxytocin 20 min, all before NSER.

	Embryo survival		
	n	24 h	48 h
G0.0	25	52.0 (13/25)	60.0 (15/25)
G0.5	24	50.0 (12/24)	54.0 (13/24)
G1.0	24	54.0 (13/24)	58.0 (14/24)

(n/n) Number of embryos.
[a,b] Within a row, means without a common superscript differed (Chi-Square test; $P < 0.05$).

Analysis of the gene expression profile of blastocysts after the NSER procedure demonstrates that the genes associated with embryonic quality (*NANOG* and *OCT-4*), cellular stress (*PDRX1* and *HSP90*), and apoptosis (*BAX* and *BCL-2*) were not affected by estradiol use (0.5 or 1.0 mg) in the cervical relaxation protocol. Crucially, however, using 100 μg estradiol benzoate, diluted in 2.5 ml of absolute ethyl alcohol, Santos et al. [12] observed a reduction in the levels of *NANOG* and *OCT4* transcripts in embryos exposed to cervical relaxation protocols with estradiol when compared to control embryos (not exposed). The disagreement between the results can be attributed to differences in the estradiol dilution method, which may have increased the drug diffusion and, consequently, its bioavailability in the uterine environment – thus, triggering side effects in the embryo. We also cannot rule out a potential deleterious effect due to the combination of estradiol benzoate with alcohol. Notably, a higher recovery rate was observed in the G0.5 compared to the G0.0 group. This may largely result from the relatively low accuracy of ultrasound for CL counting in ovaries with a high number of ovulations, as demonstrated by Pinto et al. [21]. It is important, therefore, to emphasize that this difference cannot be associated with the treatment, since the recovery rate is associated with factors prior to cervical relaxation, such as the number of oocytes ovulated and embryos fertilized.

The stress that the embryo experiences during cryopreservation is an important factor to understand, which makes this method an important tool for inferring embryonic quality. In this regard, several studies have used this strategy to estimate the quality of embryos from different sources (*in vivo* or *in vitro*) and cultured *in vitro* under different conditions. In consideration of this context, our study has demonstrated that, regardless of the dose, estradiol use in the cervical relaxation protocol does not affect embryonic survival after cryopreservation. On average, 56 % and 60 % of embryos exposed and unexposed to estradiol, respectively, survived cryopreservation. These results align with a similar result using surgically recovered embryos reported previously by our group, achieved without cervical relaxation induction [22]. Furthermore, using a concentration of 1.0 mg of estradiol in the cervical relaxation protocol, Brair et al. [23] reported an average embryo survival of 48 % after slow freezing. Hence, the evidences gathered in this study demonstrates that the use of estradiol (0.5 or 1.0 mg) in the cervical relaxation protocol does not compromise embryonic viability or the capacity for *in vitro* development after cryopreservation.

5. Conclusions

The removal of estradiol benzoate from the cervical relaxation protocol does not compromise the efficiency and duration of the non-surgical embryo retrieval procedure. Conversely, its presence in the

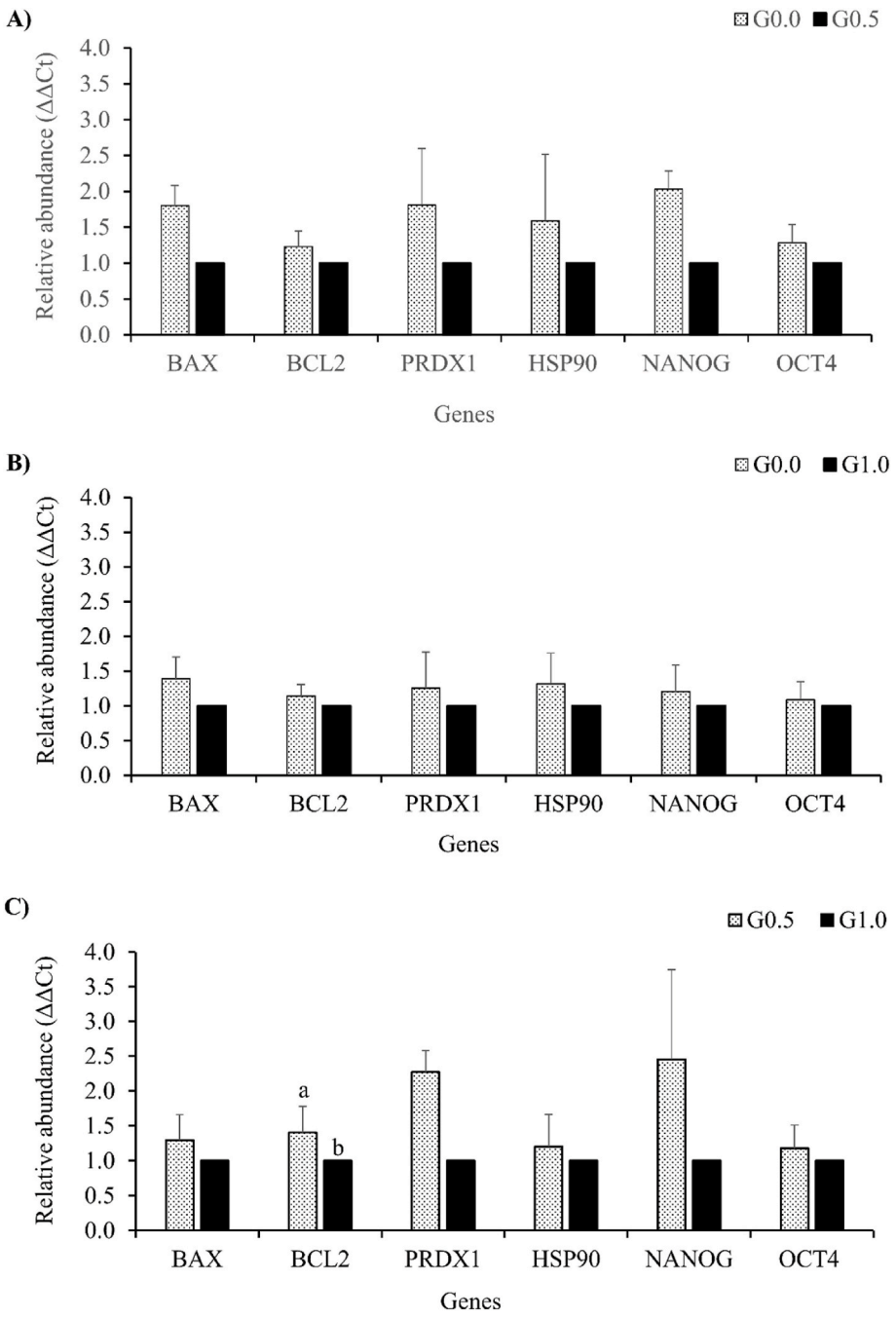

Fig. 1. Abundance of transcripts determined in blastocysts recovered from superovulated females submitted to the cervical relaxation protocol using different estradiol benzoate doses. The groups G0.0, G0.5, and G1.0 underwent cervical relaxation, respectively, with 0.0, 0.5, or 1.0 mg of estradiol benzoate associated with 37.5 μg of d-cloprostenol for 16 h and 50 IU of oxytocin 20 min, all before NSER. The different letters indicate the statistical differences ($P < 0.05$) between groups.

cervical relaxation protocol does not affect embryonic quality, survival after cryopreservation, or the expression profile of genes associated with embryonic quality (*OCT4* and *NANOG*), cell stress (*HSP90* and *PRDX1*), and apoptosis (*BCL2* and *BAX*). However, in consideration of the potential side effects on consumers, we recommend the removal of this steroid from the cervical relaxation protocol, aiming at the non-surgical recovery of sheep embryos.

CRediT authorship contribution statement

Ribrio Ivan Tavares Pereira Batista: Data curation, Methodology, Writing – original draft, Writing – review & editing. **Jenniffer Hauschildt Dias:** Data curation, Formal analysis, Writing – original draft. **Joedson Dantas Gonçalves:** Data curation, Formal analysis, Investigation, Validation. **Gabriel Brun Vergani:** Data curation, Validation. **Maria Emilia Franco Oliveira:** Data curation, Investigation, Writing – original draft. **Joanna Maria Gonçalves Souza-Fabjan:** Data curation, Methodology, Writing – original draft, Writing – review & editing. **Jeferson Ferreira Fonseca:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing.

Acknowledgements

This study was supported by CNPq (303727/2021–7), and EMBRAPA (Project Superovi – 3/20.22.03.004.00.06). The authors

would like to thank the Embrapa Gado de Leite Multi-User Bioefficiency and Sustainability Laboratory for providing animals, facilities, food, and staff.

References

[1] Dias J, Gonçalves J, Arrais A, Batista R, Souza-Fabjan J, Bastos R, et al. Single dose of 300 IU hCG in the early luteal phase in superovulated ewes: effects on corpora lutea, progesterone profile, and embryo recovery. Anim Reprod Sci 2022:107101. https://doi.org/10.1016/J.ANIREPROSCI.2022.107101.

[2] Arrais AM, Mello MRB de, Vergani GB, Figueira LM, Esteves SN, Pereira Vs do A, et al. NonSurgical embryo recovery from estrus-synchronized or superovulated morada nova ewes: a feasible strategy for sheep embryo banking. Biopreserv Biobanking 2021;00:1–9. https://doi.org/10.1089/bio.2020.0125.

[3] Dias JH, Pupin MA, Duarte GS, Brair VL, de Paula CJC, de Sousa MAP, et al. Successful transcervical uterine flushing can be performed without or reduced dose of oestradiol benzoate in cervical relaxation protocol in Dorper ewes. Reprod Domest Anim 2020;55:844–50. https://doi.org/10.1111/RDA.13692.

[4] Fonseca JF, Oliveira MEF, Brandão FZ, Batista RITP, Garcia AR, Bartlewski PM, et al. Non-surgical embryo transfer in goats and sheep: the Brazilian experience. Reprod Fertil Dev 2019;31:17–26. https://doi.org/10.1071/RD18324.

[5] Takemura M, Itoh H, Sagawa N, Yura S, Korita D, Kakui K, et al. Cyclic mechanical stretch augments hyaluronan production in cultured human uterine cervical fibroblast cells. Mol Hum Reprod 2005;11:659–65. https://doi.org/10.1093/molehr/gah229.

[6] Dias JH, Gonçalves JD, Arrais AM, Souza-Fabjan JMG, Bastos R, Batista RITP, et al. 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. Domest Anim Endocrinol 2022;82:106751. https://doi.org/10.1016/j.domaniend.2022.106751.

[7] Costine BA, Sayre BL, Inskeep EK. Embryotoxicity of regressing corpora lutea in ewes. Reproduction 2001;122:883–7. https://doi.org/10.1530/rep.0.1220883.

[8] González-Bulnes A, Baird DT, Campbell BK, Cocero MJ, García-García RM, Inskeep EK, et al. Multiple factors affecting the efficiency of multiple ovulation and embryo transfer in sheep and goats. Reprod Fertil Dev 2004;16:421–35. https://doi.org/10.1071/RD04033.

[9] Oliveira MEF, Arrais AM, Vergani GB, Esteves SN, Pereira Vs do A, Garcia AR, et al. Hormonal-induced cervical relaxation during diestrus in ewes : cervical transposing feasibility and use of hCG for rescuing disrupted luteal function hormonal-induced cervical relaxation during diestrus in ewes: cervical transposing feasibility and use o. J Appl Anim Welfare Sci 2022;00:1–17. https://doi.org/10.1080/10888705.2022.2141576.

[10] Fonseca JF, Esteves LV, Zambrini FN, Brandão FZ, Peixoto MGCD, Verneque RS, et al. Viable offspring after successful non-surgical embryo transfer in goats. Arq Bras Med Vet Zootec 2014;66:613–6. https://doi.org/10.1590/1678-41626783.

[11] Figueira LM, Alves NG, Batista RITP, Brair VL, Lima RR, Oliveira MEF, et al. Pregnancy rate after fixed-time transfer of cryopreserved embryos collected by non-surgical route in Lacaune sheep. Reprod Domest Anim 2019;54:1493–6. https://doi.org/10.1111/rda.13550.

[12] Santos JDR, Batista RITP, Ungerfeld R, Taira AR, Espírito Santo CG do, Souza-Fabjan JMG, et al. Hormonal protocol used for cervical dilation in ewes does not affect morphological embryo quality but reduces recovery rate and temporarily alters gene expression. Vet Rec 2021. https://doi.org/10.1002/VETR.1064.

[13] Mapletoft RJ, Remillard R, Lindsey BR. Chapter 9. Certification and identification of embryos. Man Int Embryo Transf Soc 2020;95–108.

[14] Fonseca JF, Batista RITP, Souza-Fabjan JMG, Oliveira MEF, Brandão FZ, Viana JHM. Freezing goat embryos at different developmental stages and quality using ethylene glycol and a slow cooling rate. Arq Bras Med Vet Zootec 2018;70:1489–96. https://doi.org/10.1590/1678-4162-10196.

[15] Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 2003;339:62–6. https://doi.org/10.1016/S0304-3940(02)01423-4.

[16] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25:402–8. https://doi.org/10.1006/METH.2001.1262.

[17] Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol Lett 2004 Mar;26(6):509–15. https://doi.org/10.1023/b:bile.0000019559.84305.47.

[18] Dias JH, Gonçalves JD, Arrais AM, Souza-Fabjan JMG, Bastos R, Batista RITP, Siqueira LGB, Oliveira MEF, Fonseca JF. 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. Domest Anim Endocrinol 2023 Jan;82:106751. https://doi.org/10.1016/j.domaniend.2022.106751.

[21] Pinto P, Bragança GM, Balaro M, Arashiro E, Dos Santos GB, de Souza GN, Souza-Fabjan J, Da Fonseca JF, Brandão FZ. Colour-Doppler ultrasound imaging as a laparoscopy substitute to count corpora lutea in superovulated sheep. Reprod Domest Anim 2018 Feb;53(1):266–9. https://doi.org/10.1111/rda.13089.

[22] Saraiva HFRA, Batista RITP, Alfradique VAP, Pinto PHN, Ribeiro LS, Oliveira CS, Souza-Fabjan JMG, Camargo LSA, Fonseca JF, Brandão FZ. l-carnitine supplementation during vitrification or warming of in vivo-produced ovine embryos does not affect embryonic survival rates, but alters CrAT and PRDX1 expression. Theriogenology 2018 Jan 1;105:150–7. https://doi.org/10.1016/j.theriogenology.2017.09.022.

[23] Brair VL, Maia ALRS, Correia LFL, Barbosa NO, Santos JDR, Brandão FZ, Fonseca JF, Batista RITP, Souza-Fabjan JMG. Gene expression patterns of in vivo-derived sheep blastocysts is more affected by vitrification than slow freezing technique. Cryobiology 2020 Aug;95:110–5. https://doi.org/10.1016/j.cryobiol.2020.05.009.

[24] Praveen Chakravarthi V, Kona SS, Siva Kumar AV, Bhaskar M, Rao VH. Quantitative expression of antiapoptotic and proapoptotic genes in sheep ovarian follicles grown in vivo or cultured in vitro. Theriogenology 2015 Mar 1;83(4):590–5. https://doi.org/10.1016/j.theriogenology.2014.10.024.

[25] Sengodan R, Sivasankaran B, Gopal D, Chitravelan V. Peroxiredoxins (PRDX 1–6) expression pattern in immature, matured oocytes and various stages of in vitro fertilized and parthenogenetically activated preimplantation ovine embryos. J Sci 2014;4:382–9.

[26] Gharibzadeh Z, Riasi A, Ostadhosseini S, Hosseini SM, Hajian M, Nasr-Esfahani MH. Effects of heat shock during the early stage of oocyte maturation on the meiotic progression, subsequent embryonic development and gene expression in ovine. Zygote 2015 Aug;23(4):573–82. https://doi.org/10.1017/S0967199414000203.

[27] Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Succu S, Berlinguer F, Ledda S. Different temporal gene expression patterns for ovine pre-implantation embryos produced by parthenogenesis or in vitro fertilization. Theriogenology 2010 Sep 15;74(5):712–23. https://doi.org/10.1016/j.theriogenology.2010.03.024.

[28] Hogg K, McNeilly AS, Duncan WC. Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine fetal ovary. Endocrinology 2011 May;152(5):2048–59. https://doi.org/10.1210/en.2010-1219.

[29] O'Connor T, Wilmut I, Taylor J. Quantitative evaluation of reference genes for real-time PCR during in vitro maturation of ovine oocytes. Reprod Domest Anim 2013 Jun;48(3):477–83. https://doi.org/10.1111/rda.12112.