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Effect of antifreeze protein I in the freezing solution on *in vivo*-derived sheep embryos

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ABSTRACT

This study evaluated the effects of different antifreeze protein type I (AFP I) concentrations added to a slow freezing solution in sheep *in vivo*-derived embryos. Good-quality embryos were allocated into: AFP-free (CONT); 0.1 μg/mL of AFP I (AFP0.1); or 0.5 μg/mL of AFP I (AFP0.5). After thawing, embryos were *in vitro* cultured (IVC) for 48 h. At 24 h and 48 h of IVC, dead cells and apoptosis, mitochondrial activity, intracellular reactive oxygen species (ROS), and glutathione (GSH) evaluations were performed. At 24 h, evaluated embryos were submitted to RT-qPCR for metabolism (*SIRT2*, *PRDX1*, *OCT4*, *CDX2*) and quality (*AQP3*, *CDH1*, *HSP70*, *BAX*, *BCL2*) genes. The *in vitro* survival rate was 56% (22/39) for CONT, 60% (32/53) for AFP0.1, and 53% (23/43) for AFP0.5 ($p > 0.05$). A tendency ($p = 0.09$) for a higher blastocyst hatching rate was noted in AFP0.1 (62%) compared to AFP0.5 (33%), and both groups were similar to CONT (50%). An increased ($p < 0.05$) mitochondrial activity at 24 h was observed in AFP0.1 compared to CONT. No differences ($p > 0.05$) were observed in oxidative stress homeostasis and viability between treatments. A downregulation ($p < 0.05$) of *CDH1* in AFP0.1 and a downregulation of AQP3 in AFP0.5 were observed in comparison to the other groups. An upregulation ($p < 0.05$) was detected in *HSP70* and *BCL2* on AFP0.5 compared to AFP0.1 group. The addition of AFP I in slow freezing solution can benefit cryopreserved sheep *in vivo*-derived embryos, without affecting embryonic survival.

1. Introduction

Multiple ovulation followed by embryo transfer (MOET) and *in vitro* embryo production (IVEP) are assisted reproduction techniques (ARTs) applied to livestock genetic improvement. In small ruminants, the IVEP outcomes are generally similar to those found in cattle, but an improvement in embryonic survival after cryopreservation has been reached in MOET (Souza-Fabjan et al., 2023). The association of cryopreservation with those ARTs has several advantages (Sudano et al., 2013), but the success of embryo cryopreservation can be affected by several cryoinjuries leading to lower pregnancy rates when compared to the transfer of fresh embryos (Marsico et al., 2019).

Most of these cryoinjuries are derived from intra- and extracellular ice crystal formation that can disrupt cell membranes and organelles on slow freezing, and from cryoprotectant concentrations that can induce osmotic stress as well cytotoxic damages on vitrification (Wolkers and Oldenhof, 2021). Slow freezing is the main cryopreservation technique for *in vivo*-derived sheep embryos (Menchaca et al., 2016), with a potential for a higher survival and pregnancy rate compared to vitrification (Figueira et al., 2019). Both cryopreservation methods result in similar ultrastructural lesions (Bettencourt et al., 2009), however, the cryoinjuries of slow freezing compared to vitrification turn out to be less harmful molecularly on embryos, presenting gene expression modulation more similar to fresh embryos (Brair et al., 2020).

The cryobiologist community, aiming to improve cryopreservation outcomes, has been testing several substances with variable results (Raju et al., 2021). Antifreeze proteins (AFPs), a subgroup of ice-binding proteins, have been applied for the cryopreservation of different reproductive cells over the last three decades (Correia et al., 2021). Diverse biological and recombinant types of AFPs act as extracellular protectant agents (Bar-Dolev et al., 2020), and share the basic mechanism of interaction with ice crystals: thermal hysteresis – reducing the freezing point below the melting point; and ice recrystallization inhibition (Kim et al., 2017). Overall, the addition of distinct AFP did not compromise mammalian chilled (Baguisi et al., 1997; Ideta et al., 2015) or vitrified (Liang et al., 2017; Li et al., 2020) embryos. In addition,

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bovine embryos submitted to vitrification with 1 mM of antifreeze glycoprotein 8 (AFGP8) (Liang et al., 2017), and ovine embryos submitted to vitrification with 10 μg/mL of AFP derived from *Anatolia polita* (ApAFP914) (Li et al., 2020), presented enhanced mitochondrial potential and embryo re-expansion rate. Moreover, 1 mg/mL of AFP I was beneficial to sheep embryos stored in chilling conditions (Baguisi et al., 1997). However, the outcomes may differ due to cryopreservation technique, AFP type, and concentration applied, and the knowledge about the AFP addition to the embryo freezing solutions remains not fully elucidated (Correia et al., 2021). The AFP I compared to other types could result in better outcomes based on molecule size, structure, and moderate functions (Kim et al., 2017). We hypothesized that adding AFP I could improve embryo cryotolerance, reflecting an increased survival rate, viability, metabolic, and molecular gene expression pattern. Thus, this study aimed to evaluate the effects of different concentrations of AFP I added in the freezing solution of *in vivo*-derived sheep embryos on embryo viability after slow freezing/thawing.

2. Materials and methods

2.1. Ethics, location, and experimental conditions

This study was conducted under the approval of the Ethics Committee for the Use of Animals of the Universidade Federal Fluminense (#5956101218/2019), and procedures followed the ethical principles of the Brazilian Society of Science in Laboratory Animals. The research was conducted in the non-breeding season (November to December 2021) at Unidade de Pesquisa em Caprinos e Ovinos (UniPECO) in Cachoeiras de Macacu (22° 27′ S, 42° 39′ W), Rio de Janeiro, Brazil. Santa Inês ewes (4.1 ± 1.1 years old; 47.6 ± 7.6 kg; 3.0 ± 0.3 of body condition score on a scale of 1–5; Mean ± SEM) were subjected to clinical and ultrasound evaluations to confirm the absence of any reproductive or clinical disorders. Ewes were kept in an intensive system, fed with chopped Napier grass (*Pennisetum purpureum* cv. *Cameron*), 300 g of concentrate (16% of crude protein) daily, and *ad libitum* mineral salt and water.

2.2. Reagents

Unless otherwise indicated, all reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA). The AFP I (purified from *Myoxocephalus scorpius*) was obtained from A/F Protein Inc. (Waltham, MA, USA).

2.3. Experimental design

A total of 135 *in vivo*-derived viable ovine embryos [10 morulas (Mo), 44 compact morulas (Mc), 20 early blastocysts (eBl), 18 blastocysts (Bl), 38 expanded blastocysts (Bx), and 5 Hatched blastocysts (HBl)] were allocated into three groups, with different AFP I concentrations, added to the freezing medium: AFP-free (CONT; $n = 39$; 3 Mo, 16 Mc, 4 eBl, 4 Bl, 11 Bx, 1 HBl); 0.1 μg/mL of AFP I (AFP0.1; $n = 53$; 4 Mo, 15 Mc, 7 eBl, 8 Bl, 16 Bx, 3 HBl); and 0.5 μg/mL of AFP I (AFP0.5; $n = 43$; 3 Mo, 13 Mc, 9 eBl, 6 Bl, 11 Bx, 1 HBl). The distribution was carried out by convenience sampling, considering the number of embryos at each development stage recovered from each sheep, to obtain a similar number of embryonic stages in the groups. The minimum sample size required per group was calculated through the software G*Power v3.1 (Faul et al., 2007), assuming $\alpha = 0.05$, 80% of power, and effect size $d = 0.5$. After thawing, the embryos were cultured *in vitro* in SOFaa medium (Holm et al., 1999), at 38.5 °C, 5% CO_2, and 5% O_2 for 48 h in a benchtop incubator. At 24 h of embryo culture, 15 re-expanded (viable) blastocysts from each treatment were removed from *in vitro* culture and, in groups of five embryos, subjected to mitochondrial activity evaluation, intracellular reactive oxygen species (ROS) and glutathione (GSH) levels analysis, and dead cells and apoptosis assay. After these evaluations, the same blastocysts ($n = 15$/treatment) were allocated in three pools of five blastocysts, and frozen on cryotubes (RNase and DNase-free) at −196 °C until molecular analysis. The RT-qPCR was performed for transcripts related to embryo metabolism and quality (*SIRT2*, *PRDX1*, *CDH1*, *AQP3*, *OCT4*, *CDX2*, *HSP70*, *BAX*, *BCL2*), and *GAPDH* and *ACTB* were used as housekeeping genes. After 48 h of culture, the remaining *in vitro* cultured embryos were separated regarding their viability/survivability and both viable and non-viable groups from each treatment were subjected to the same metabolic evaluations. The survival rate was assessed at 24 and 48 h, while the blastocyst hatching rate was only at 48 h.

2.4. Embryo recovery and classification

Thirty-five ewes were synchronized and superovulated as reported by Taira et al. (2022). Multiparous ewes were synchronized by the use of intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Progespon®, Syntex, Buenos Aires, AR) during six days, plus 0.24 mg of cloprostenol (Estron®, Agener União Saúde Animal, Embu-Guaçu, SP, BR) and 300 IU of eCG (Novormon 5000; MSD Animal Health, São Paulo, SP, BR) were administered intramuscularly (IM), one day before sponge withdrawal. Thirty-six hours after sponge removal, 0.025 mg of lecireline (Gestran Plus®; Tecnopec, São Paulo, SP, BR) was administered IM, and the superovulation began 80 h after the intravaginal sponge removal. Ewes received 133 mg of pFSH (Folltropin-V®, Bioniche Animal Health, Belleville, CA) IM, divided into six decreasing doses (25, 25, 15, 15, 10, 10%) every 12 h. Simultaneously with the first dose of pFSH, an intravaginal device of progesterone (P4; 0.36 g; Primer PR, Agener União Saúde Animal, Embu-Guaçu, SP, BR) was inserted into all ewes, remaining *in situ* until the fifth dose of pFSH. Cloprostenol sodium was given twice, with the last dose of pFSH and after 24 h the ewes received lecireline. All ewes were checked for estrous behavior and mated naturally with five fertile rams (7:1) every 12 h, from the sixth dose of pFSH until the end of estrus. The average number of corpora lutea was 9.4 ± 0.9 (Mean ± SEM). On the sixth day after natural mating, a cervical dilation protocol was performed (Leite et al., 2018), and embryos were retrieved by non-surgical embryo recovery (NSER) (Souza-Fabjan et al., 2023), with a recovery rate of 50%. All recovered structures were transferred to a holding medium (*Phosphate-buffered saline* [PBS] supplemented with 0.4% bovine serum albumin [BSA] fraction V) and classified according to their developmental stage (Mo, Mc, eBl, Bl, Bx, HBl) and quality. Only Grade I (excellent) and II (good) embryos were used, and those classified as Grades III (bad) and IV (degenerated) were discarded (Mapletoft et al., 2020).

2.5. Slow freezing and thawing

Slow freezing procedures were performed according to Brair et al. (2020). The AFP I concentrations were previously chosen according to Correia et al. (2021). The slow freezing medium was ethylene glycol (EG; 1.5 M) in PBS with different concentrations of AFP I: 0 μg/mL (CONT); 0.1 μg/mL (AFP0.1); or 0.5 μg/mL (AFP0.5). The 0.25 mL sterile straws (IMV Technologies, Paris, FR) were completed with a base solution of PBS supplemented with 20% fetal bovine serum interspersed with air bubbles. Freezing was performed in a freeze control machine (Freeze Control CL-5000®, Cryologic, Victoria, AU), 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 and then, holding for 10 min at −32 °C, until plunging into liquid N_2 for storage for six months. Thawing was performed at room temperature for 5 s followed by a water bath at 37 °C for 30 s. After thawing, embryos were kept in HEPES SOFaa medium for <1 h (BIOK HSOF, Bioklone Reprodução Animal, Passo Fundo, RS, BR).

2.6. In vitro culture (IVC)

After thawing, embryos of each group were *in vitro* cultured

according to their developmental stage in each well, 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 benchtop incubator (EVE, WTA, Cravinhos, SP, BR). The embryo survival rate after thawing was recorded at 24 and 48 h according to blastocele re-expansion and embryo development. The morula development rate was evaluated at 48 h, based on the total morulas (morulas + compact morulas) that developed to the blastocyst stage. The total blastocyst hatching rate was recorded at 48 h based only on blastocysts that reached hatching, and those already hatched at thawing were not considered in this rate.

2.7. Epifluorescence microscopy

2.7.1. Mitochondrial activity

Analysis was performed according to Xu et al. (2019) with slight modifications, using MitoTracker Green FM (Invitrogen, Waltham, MA, USA), excitation/emission (ex-em) 490/516 nm, following dilution manufacturer's instructions. Briefly, the blastocysts were incubated in a 100 μL HSOF drop containing 250 nM of Mitotracker Green FM at 38.5 °C for 30 min. Then, they were stained with 1 μg/mL of Hoechst 33342, ex-em 350/461 nm, for 5 min and washed twice with PBS plus 0.4% BSA. Each embryo was placed in an individual drop of 2 μL, and the analysis was performed under an epifluorescence microscope at the same moment (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with appropriate filter set and image capture system (Pylon viewer, Basler AG, Exton, PA, USA).

2.7.2. Intracellular reactive oxygen species (ROS) and glutathione (GSH) levels

Analysis was performed according to Sánchez-Ajofrín et al. (2020) with 2′,7′-dichlorodihydrofuorescein diacetate (H_2DCFDA), ex-em 485/535, used to detect the ROS levels, and CellTracker Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF_2HC, ex-em 371/464 nm, used to detect the GSH levels, following manufacturer's instructions (Invitrogen, Waltham, MA, USA). Briefly, the blastocysts were incubated in a 100 μL HSOF drop containing 10 μM of H_2DCFDA and 10 μM of CMF_2HC in the dark for 30 min at 38.5 °C. Then, embryos were washed twice with PBS plus 0.4% BSA, and each embryo was placed in an individual drop of 2 μL. The analyses were performed under an epifluorescence microscope at the same moment (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with the appropriate filter set and image capture system (Pylon viewer, Basler AG, Exton, PA, USA).

2.7.3. Dead cell and apoptosis assay

The assay was performed using Annexin V Alexa Fluor 488 conjugate (Invitrogen, Waltham, MA, USA), ex-em 491/516 nm, and Propidium Iodide (PI), ex-em 535/615 nm, following manufacturer's instructions. The blastocysts were incubated in a 100 μL HSOF total drop containing 5 μL of Annexin V, and 1 μL of Propidium Iodide (100 μg/mL) at 38.5 °C for 15 min. Then, they were labeled with 1 μg/mL of Hoechst 33342 for 5 min and washed twice with PBS plus 0.4% BSA. Each embryo was placed in an individual drop of 2 μL, and the analysis was performed under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with the appropriate filter set and image capture system at the same moment (Pylon viewer, Basler AG, Exton, PA, USA). The cells were classified as: viable – cells not stained in green and red; apoptotic – cells showing green staining; dead – cells showing red staining (Sánchez-Ajofrín et al., 2020). The total cell number was obtained by DNA blue staining by Hoechst 33342.

2.7.4. Recovery data of images

Each image was taken with the same parameters. All images obtained of mitochondrial activity, ROS, and GSH were analyzed using the ZEN lite 3.1 version (ZEISS, Oberkochen, BW, DEU). The color channels were converted to grayscale, then embryos were measured to obtain the area (μm^2), fluorescence intensity means (arbitrary units, A.U.), and integrated density (area of selected embryo x fluorescence intensity mean). The following formula was used to obtain the corrected total fluorescence (CTF): CTF = integrated density – (area of selected embryo × mean fluorescence of background readings). The mitochondrial activity, ROS, and GSH levels were calculated by the following formula: fluorescence intensity = corrected total fluorescence/area of the selected embryo (Qu et al., 2020). The relative fluorescence intensity ratio was calculated by adjusting the value obtained for the CONT group at 24 h as a reference. For dead cells and apoptosis assay, the cell counting was performed in QuPath 0.3.2 (Quantitative Pathology & Bioimage Analysis, University of Edinburgh, Edinburgh, Sld, GBR). The color channel images for each embryo per assay were also split-merged.

2.8. Gene expression

2.8.1. RNA extraction

At 24 h of IVC, after epifluorescence microscopy evaluations, blastocysts (n = 15/treatment) were allocated into three pools of five blastocysts, and frozen on cryotubes (RNase and DNase-free) with a minimal amount of medium at −196 °C until molecular analysis, since fluorescent probes have no impact on the gene expression analysis (Yaron et al., 2014). Total RNA was extracted using the RNeasyMicro Kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions, and treated with DNase I provided in the same kit. 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: CONT – 5.8 ng/μL; AFP0.1–7.3 ng/μL; AFP0.5–7.3 ng/μL) on a spectrophotometer (Nanodrop Lite, Thermo Fisher Scientific, Wilmington, DE, USA).

2.8.2. Reverse transcription

For reverse transcription, the SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), was used for all samples in the same RNA concentration (34 ng per sample). 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); totalizing a final volume of 20 μL. Each sample in the first mix was incubated at 65 °C for 5 min, followed by 4 °C for 3 min, when the second mix was added. Afterwards, the cDNA synthesis was followed by 50 °C for 10 min, 80 °C for 10 min, and finally, 4 °C for 1 min, and then samples were chilled on ice or stored at −20 °C until quantitative polymerase chain reaction (qPCR).

2.8.3. 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 10 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 for each reaction (Ramakers et al., 2003). The primer efficiency average was: 1.84 to *SIRT2* (sirtuin 2); 1.95 to *PRDX1* (peroxiredoxin 1); 2.06 to *BAX* (BCL2 associated X); 1.83 to *BCL2* (B-cell lymphoma protein 2); 2.03 to *OCT4* (octamer-binding transcription factor 4); 1.90 to *CDX2* (caudal type homeobox 2); 2.01 to *HSP70* (70 kilodalton heat shock protein); 1.93 to *AQP3* (aquaporin 3); 2.00 to *CDH1* (cadherin-1); 1.98 to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase); and 1.89 to *ACTB* (beta-

Table 1
Sequences of primers used in the gene expression analysis of *in vivo*-derived sheep embryos submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I).

Gene symbols	Primer sequences (5′ to 3′)	Amplicon size (bp)	References
ACTB	F: GGG AAA TCG TGC GTG ACA TTA AG R: TGT GTT GGC GTA AGG TCT TTG	273	(Sharma et al., 2012)
AQP3	F: GGG TGC CCA TTG TCT CTC C R: CAA CTT CAC ATT CTC CTC GTC	119	(Bebbere et al., 2010)
BAX	F: CCT GGG ATC TTG AAA CTC TCC TT R: CTG AGC CAG GCT GAA ATC AAA A	566	(Chakravarthi et al., 2015)
BCL2	F: GCC GAG TGA GCA GGA AGA C R: GTT AGC CAG TGC TTG CTG AGA	214	(Chakravarthi et al., 2015)
CDH1	F: TGT GAC TGT GAT GGG ATC GT R: ACC CTT CTC CTC CGA ACA AG	155	(Bebbere et al., 2010)
CDX2	F: GCC ACC ATG TAC GTG AGC TAC R: ACA TGG TAT CCG CCG TAG TC	140	(Sakurai et al., 2010)
GAPDH	F: ATG TTT GTG ATG GGC GTG AA R: ACA GTC TTC TGG GTG GCA GT	176	(O'Connor et al., 2013)
HSP70	F: AAC ATG AAG AGC GCC GTG GAG G R: GTT ACA CAC CTG CTC CAG CTC C	169	(Lacetera et al., 2006)
OCT4	F: GAG GAG TCC CAG GAC ATC AA R: CCG CAG CTT ACA CAT GTT CT	204	(Bebbere et al., 2010)
PRDX1	F: CAA AGC AAC AGC TGT TAT GC R: GAG AAT CCA CAG AAG CAC C	197	(Sengodan et al., 2014)
SIRT2	F: GCC AGA CTG ACC CTT TCC TC R: GGT GGT GGA GAA TTC CTG GG	253	(Ramos-Ibeas et al., 2022)

actin).

2.9. Statistical analysis

The normal distribution of all variables was determined by Shapiro-Wilk test, and homoscedasticity by Levene test. The variables with residues normally distributed (mitochondrial activity, apoptotic, dead, and viable cells) were analyzed with a one-way analysis of variance (ANOVA) followed by the Tukey test while those not normally distributed (ROS, GSH, ROS/GSH) were analyzed by Welch test followed by Games-Howell test. The embryo survival rate, morulas development rate, and total hatching rate were compared by Pearson chi-square (χ^2) test. All analyses were performed in IBM SPSS version 25, and a value of $p < 0.05$ was considered statistically significant, and a value of $0.05 < p \leq 0.10$ was considered statistical tendency. In the statistical analysis of gene expression, relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software (Livak and Schmittgen, 2001). The expression of each target gene was normalized using the geometric mean of *GAPDH* and *ACTB* values. The values of Pearson correlation coefficient observed for the *GAPDH* ($r^2 = 0.893$) and *ACTB* ($r^2 = 0.760$) genes demonstrate stability ($p < 0.01$) of these reference genes using the BestKeeper – Excel tool (Pfaffl et al., 2004). The gene expression results are presented as Mean ± SEM.

3. Results

3.1. In vitro culture (IVC)

The *in vitro* survival rate after thawing was similar ($p > 0.05$) in frozen embryos with different concentrations of AFP I, both at 24 and 48 h of IVC. When data were pooled regardless of the treatment, the overall survival rate at 24 and 48 h was 49% and 57%, respectively. The AFP0.5 embryos tended ($p = 0.09$) to have lower blastocyst hatching rate compared to AFP0.1, but both groups did not differ to CONT. The results of embryo survival and hatching rates are shown in Table 2. The morula development after 48 h of IVC was 16% (3/19) in CONT, 16% (3/19) in AFP0.1, and 19% (3/16) in AFP0.5 ($p > 0.05$).

3.2. Epifluorescence microscopy

3.2.1. Mitochondrial activity

Regarding mitochondrial activity, re-expanded blastocysts at 24 h of IVC from the AFP0.1 group had an increased mitochondrial activity compared to the CONT group ($p < 0.05$). Nonetheless, at 48 h of IVC, this difference was not observed in the viable embryos among the three groups ($p > 0.05$). Interestingly, in the CONT group, the viable embryos presented a higher mitochondrial activity ratio at 48 h compared to those evaluated at 24 h of IVC ($p < 0.05$), while both AFP groups did not show this pattern ($p > 0.05$). The mitochondrial activity fluorescence intensity is shown in Fig. 1A.

3.2.2. Intracellular reactive oxygen species (ROS) and glutathione (GSH) levels

Upon the oxidative stress evaluations, the fluorescence intensity of ROS levels did not present differences ($p > 0.05$) among groups at 24 or 48 h of IVC. Similarly, no differences ($p > 0.05$) were observed among treatments at 24 or 48 h of IVC for GSH levels fluorescence intensity. However, a reduction ($p < 0.05$) of ROS fluorescence intensity was observed in viable embryos of both AFP groups at 48 h compared to those of 24 h (Fig. 1B), and for GSH levels (Fig. 1C). The ratio of ROS per GSH was also similar ($p > 0.05$) among treatments at 24 or 48 h of IVC,

Table 2
Survival, morulas development, and hatching rates of *in vivo*-derived sheep embryos previously submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 48 h after thawing.

Group	Embryo survival rate at 24 h after thawing (%)	Total embryo survival rate (%)	Total of morulas development (%)	Total hatching rate/Total of viable blastocysts[1] (%)
CONT[#]	19/39 (49)	22/39 (56)	3/19 (16)	11/22 (50)
AFP0.1[†]	28/53 (53)	32/53 (60)	3/19 (16)	21/34 (62)
AFP0.5[‡]	19/43 (44)	23/43 (53)	3/16 (19)	9/27 (33)[*]
TOTAL	66/135 (49)	77/135 (57)	9/54 (17)	41/83 (49)

[#] CONT = Control group, which contained - 3 morulas, 16 compact morulas, 4 early blastocysts, 4 blastocysts, 11 expanded blastocysts, 1 hatched blastocyst.
[†] AFP0.1 = 0.1 μg/mL of AFP I group, which contained – 4 morulas, 15 compact morulas, 7 early blastocysts, 8 blastocysts, 16 expanded blastocysts, 3 hatched blastocysts.
[‡] AFP0.5 = 0.5 μg/mL of AFP I group, which contained – 3 morulas, 13 compact morulas, 9 early blastocysts, 6 blastocysts, 11 expanded blastocysts, 1 hatched blastocyst.
[1] Only viable blastocysts were considered to calculate the hatching rate (morulas and compact morulas that blocked their development, and blastocysts already hatched before cryopreservation were not considered for the hatching rate).
[*] Tendency for a lower hatching rate in AFP0.5 compared to AFP0.1 ($p = 0.09$).

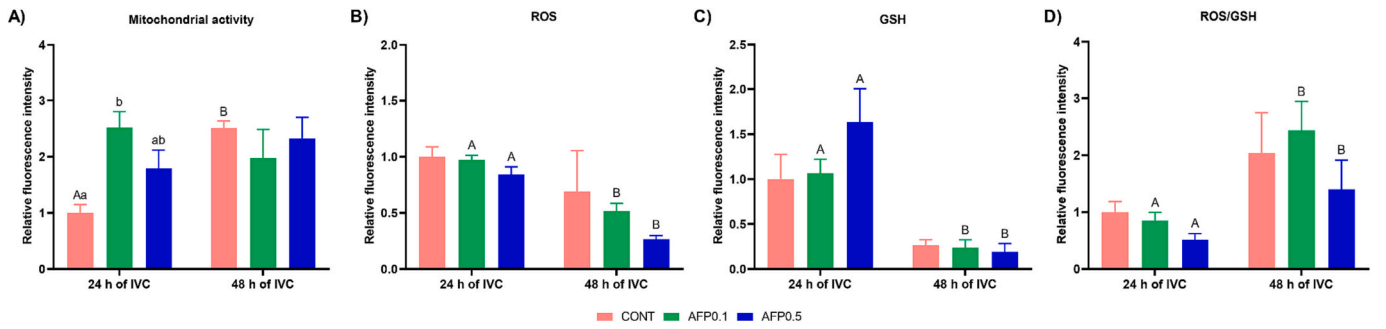

Fig. 1. Relative fluorescence intensity levels (Mean ± SEM of arbitrary units) of A) Mitochondrial activity (MitoTracker Green FM), B) intracellular reactive oxygen species (ROS, H_2DCFDA), C) glutathione (GSH, CMF_2HC), and D) ROS/GSH ratio on *in vivo*-derived sheep embryos submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I), thawed and *in vitro* cultured (IVC) for 24 h or 48 h. Values with different letters differ significantly for each endpoint ($p < 0.05$): A, B differs in each group (CONT, AFP0.1 or AFP0.5) at different moments (24 *vs.* 48 h of IVC); a, b differs between groups (CONT, AFP0.1, and AFP0.5) at the same time-point (24 h or 48 h of IVC). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

but an increase ($p < 0.05$) in this ratio was observed in viable embryos of AFP groups at 48 h compared to those of 24 h (Fig. 1D).

3.2.3. Dead cell and apoptosis assay

The cell viability assessed by apoptotic and dead cells assay was similar ($p > 0.05$) among treatments at any time point. In the apoptosis index, a tendency ($p = 0.09$) to increase apoptotic cells was observed in the CONT group at 48 h compared to 24 h of IVC. In the dead cells index, a tendency ($p = 0.06$) to increase dead cells was observed in the AFP0.1 group at 48 h compared to 24 h of IVC. A reduction ($p < 0.05$) in the viable cells index was observed in CONT and AFP0.5 groups at 48 h compared to 24 h of IVC, while the AFP0.1 group presented a tendency to viable cells reduction ($p = 0.09$). The results of viable, apoptotic, and dead cells are presented in Fig. 2. Representative epifluorescence staining of frozen-thawed *in-vivo* derived sheep embryos is shown in Fig. 3.

3.3. Gene expression

Embryo gene expression in the three groups is shown in Fig. 4. A downregulation ($p < 0.05$) was observed in *CDH1* abundance transcripts of AFP0.1 compared to CONT and AFP0.5 groups, and in *AQP3* abundance transcripts of AFP0.5 compared to CONT and to AFP0.1 groups. An upregulation ($p < 0.05$) was observed in *HSP70* and *BCL2* abundance transcripts of AFP0.5 compared to the AFP0.1 group. The expression of *SIRT2*, *PRDX1*, *OCT4*, *CDX2*, and *BAX* genes was similar among the aforementioned groups.

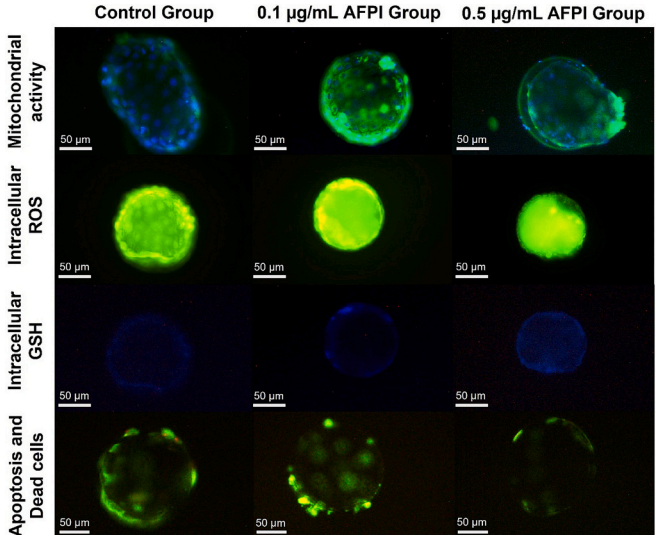

Fig. 3. Representative images of *in vivo*-derived sheep embryos submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h after thawing stained to either: mitochondrial activity (MitoTracker Green FM) merged with DNA staining (Hoechst 33.342); intracellular reactive oxygen species (ROS, H_2DCFDA); intracellular glutathione (GSH, CMF_2HC); or apoptosis and dead cells (Annexin V Alexa Fluor 488 conjugate and Propidium Iodide). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

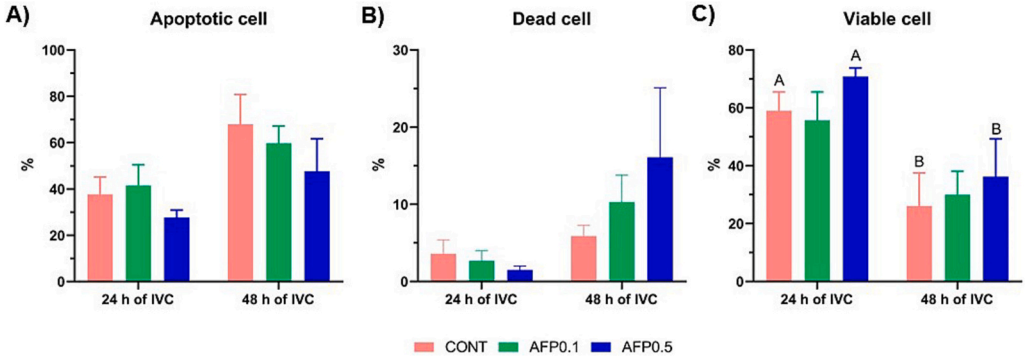

Fig. 2. Apoptotic cell, dead cell, and viable cell indexes (Mean ± SEM) at 24 and 48 h of *in vitro* culture (IVC) after thawing of *in vivo*-derived sheep embryos submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I): A, B differs in each group (CONT, AFP0.1 or AFP0.5) at different moments (24 *vs.* 48 h of IVC).

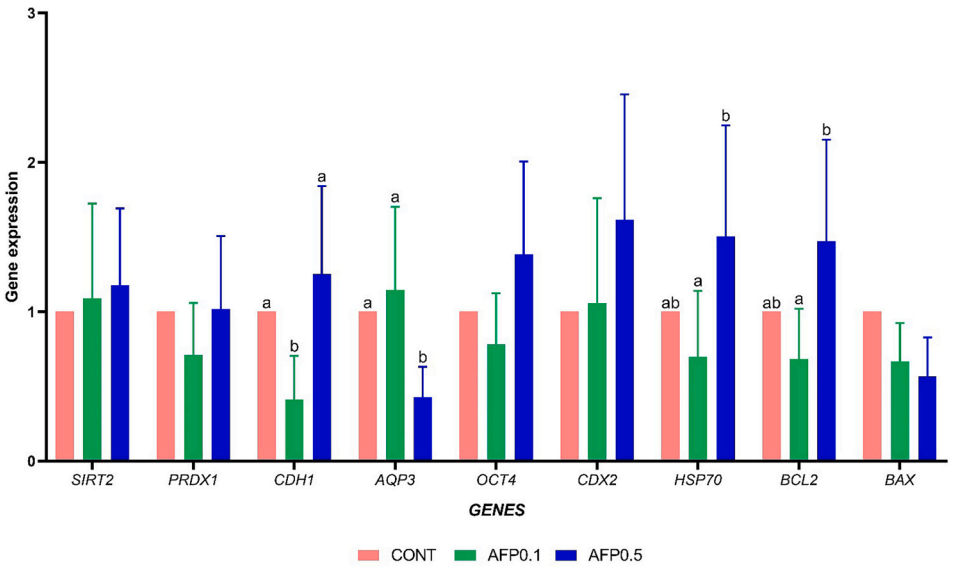

Fig. 4. Gene expression (Mean ± SEM) of sirtuin 2 (*SIRT2*), peroxiredoxin 1 (*PRDX1*), cadherin-1 (*CDH1*), aquaporin 3 (*AQP3*), octamer-binding transcription factor 4 (*OCT4*), caudal type homeobox 2 (*CDX2*), 70 kilodalton heat shock protein (*HSP70*), BCL2 associated X (*BAX*), and B-cell lymphoma protein 2 (*BCL2*) on *in vivo*-derived sheep embryos submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h after thawing. Different letters show statistical differences ($p < 0.05$).

4. Discussion

This study investigated the effects of different concentrations of AFP type I added in the freezing solution to cryopreserve *in vivo*-derived sheep embryos. Baguisi et al. (1997) have shown that 1 mg/mL of AFP I was beneficial for chilled sheep embryos at 0° to 4 °C for four days before *in vivo* transfer. More recently, the use of 10 μg/mL of ApAFP914 added during vitrification increased the hatching rate of slow-growing sheep embryos (Li et al., 2020). Nevertheless, the type and concentration of AFP used may vary depending on the cryopreservation technique (Correia et al., 2021). To the best of our knowledge, this is the first report of the addition of AFP I in slow freezing solution for sheep embryo cryopreservation. Our results pointed out that AFP I does not affect the embryo survival rate, morulas development rate, oxidative stress, and embryonic viability indexes. However, we observed higher mitochondrial activity in the AFP0.1 group at 24 h of IVC, a tendency to a higher hatching rate on AFP0.1 compared to the AFP0.5 group, and punctual gene expression modulation in both AFP treatments.

In the present study, the AFP0.1 group presented higher mitochondrial activity compared to the CONT group after 24 h of IVC. In addition, an increase in mitochondrial activity was observed in embryos of the CONT group when comparing 24 *vs.* 48 h of IVC, which was not observed in the AFP groups. This result can represent a protective effect of AFP supplementation during embryo cryopreservation on the mitochondrial activity pattern. According to Dalcin et al. (2013), thawed or warmed sheep embryos did not present mitochondrial activity after 1 h of IVC when compared to fresh embryos, which showed an intense mitochondrial activity pattern distributed throughout the cytoplasm cells. During early embryo development, a higher energy amount is required to increase biosynthesis, cell proliferation, cavitation, and hatching (Milazzotto et al., 2020). Most of the ATP energy production will be from oxidative phosphorylation, the final step of the aerobic glycolytic pathway in mitochondria (Deluao et al., 2022), that will be used by the Na^+/K^+-ATPase pump in blastocyst development (Houghton et al., 2003). Moreover, mitochondria disruption is associated with oxidative stress throughout cryopreservation (Gualtieri et al., 2021), and their activity is considered a parameter to assess the embryo quality as a viability and competence marker (Van Blerkom, 2009). The AFPs during cryopreservation of reproductive cells presented the capacity to maintain mitochondrial activity (Correia et al., 2021). On bovine vitrified blastocysts, the AFGP8 was described to benefit the mitochondrial activity after warming compared to untreated embryos (Liang et al., 2016). These facts suggest that the addition of 0.1 μg/mL of AFP I in the slow freezing medium for sheep embryos supports the mitochondrial activity after thawing.

Although an increased mitochondrial activity was observed at 24 h of IVC in the AFP0.1 group, we did not observe a reduction of intracellular ROS, GSH, and ROS per GSH ratio in this group compared to CONT in both time points evaluated. Nevertheless, the mitochondrial activity did not raise the intracellular ROS levels at 48 h of IVC, supporting mitochondrial metabolism activeness. Considering that oxidative stress is one of the factors that affect cell viability during cryopreservation (Len et al., 2019), the exacerbated ROS generation could overwhelm the cell defense capacity leading to mitochondrial disruption (Maharjan et al., 2014), and arresting the development (Milazzotto et al., 2020). On the other hand, the biological levels of ROS and antioxidant compounds (*e.g.*: GSH) are necessary for embryo development, in which ROS levels increase together with mitochondrial activity due to oxidative phosphorylation (Deluao et al., 2022). Although GSH is the major non-enzymatic defense for ROS (Deluao et al., 2022), this compound is abundantly found in the cell cytoplasm and produced in the pentose phosphate pathway (Milazzotto et al., 2020), not being directly related to the oxidative phosphorylation in mitochondria. In bovine, the addition of 0.1 mg/mL of *Leucosporidium*-derived ice-binding protein (LeIBP) to vitrification solution provides a significant reduction of ROS levels in warmed oocytes as well as enhanced the survival rate of vitrified blastocysts (Sun et al., 2020). Similarly, the use of 1 mM of AFGP8 on the vitrification of bovine oocytes provided a reduction in ROS levels after warming and increased the subsequent embryo development (Liang et al., 2016). Altogether, our results of ROS and GSH levels showed that 0.1 μg/mL of AFP I in a slow freezing medium does not affect oxidative stress but improves the mitochondrial activity in sheep embryos. These findings allow us to suggest that embryos in this group will have a greater window to development, hatching, and implantation based on a greater ATP production without redox balance compromised, allowing the Na^+/K^+-ATPase pump function and embryo metabolism.

Our results showed that dead cell and apoptosis indexes in AFP groups did not differ compared to CONT in both evaluated time points. Therefore, a decrease in viable cells index in CONT and AFP0.5 group at 48 h compared to 24 h was observed. Apoptosis is an important embryo

development regulator, and this energy-dependent process is characterized by morphological features such as cell shrinkage and blebbing, DNA fragmentation, and apoptotic body formation (Vining et al., 2021). This process also participates in cell proliferation balance by enabling the proliferation of healthy cells, preventing transmission of the altered genome in damaged cells or cells with altered metabolism patterns allowing these cells to progress to death (Ramos-Ibeas et al., 2020). Little is known about the possible protection of AFPs during embryo cryopreservation regarding apoptosis index in blastomere viability. In cryopreserved zebrafish embryos, 10 mg/mL of AFP I on vitrification solution provided higher blastomeres viability after warming (Martínez-Páramo et al., 2009). According to our viability results it is not possible to infer the best concentration tested on sheep embryos, however, their addition in slow freezing medium does not compromise embryonic viability.

Regarding gene expression, few modulations in mRNA transcript abundance were observed between groups. Marsico et al. (2020) reported that transcriptional profile competencies are affected by embryo cryosurvival, bringing on distinct molecular pathways in embryos with low cryotolerance compared to those with high cryotolerance. In the AFP0.1 group, a downregulation of *CHD1* was observed compared to CONT and AFP0.5 groups. The *CDH1* gene is responsible for coding the E-cadherin, which participates in cell-cell communication and the establishment of embryonic stem cell pluripotency (Alotaibi, 2023). The downregulation of this gene is considered to be one of the triggers to promote a partial epithelial-to-mesenchymal transition (Aban et al., 2021), and for embryo implantation success in the uterus (Varghese et al., 2021; Yamada et al., 2022). Therefore, the AFP0.1 group would be the one that could have the higher embryo implantation response based on gene expression.

In the AFP0.5 group, a downregulation of *AQP3* compared to CONT and AFP0.1 groups was observed. The *AQP3* is an aquaglyceroporin that plays a role in water homeostasis by mediating the water and cryoprotectants' movement and appears to be related to embryo apoptosis throughout cryopreservation (Kuzmany et al., 2011; Yamaji et al., 2011). The downregulation of *AQP3* has been shown to impair the functions of cells and inhibit embryo development (Xiong et al., 2013). In this context, the downregulation of *AQP3* in the AFP0.5 group may represent a deleterious effect on embryonic development. Moreover, an upregulation of *HSP70* and *BCL2* abundance transcripts was observed in AFP0.5 compared to the AFP0.1 group. The heat-shock protein 70, a chaperone encoded by *HSP70* gene, is one important cryopreservation stress marker that was already described to be upregulated in bovine embryos exposed to higher cryoprotectant concentrations (Yodrug et al., 2020). The *BCL2* is an anti-apoptotic gene that interacts with the pro-apoptotic *BAX* gene to counteract the molecular apoptosis induction (Brair et al., 2020). These genes could be related to preventing apoptosis (Mishra et al., 2017; Stamperna et al., 2021). Thus, 0.5 μg/mL of AFP I could not be the best concentration choice for slow freezing ovine embryos due to the presence of some stress factors despite maintaining embryonic viability. According to Correia et al. (2021), higher concentrations of AFPs could promote lesser benefits than lower concentrations due to dose-related negative effects, such as changes in ice crystal pattern. However, the role of AFP on sheep embryo cryopreservation has still not been fully elucidated and further investigations are required to enhance cryopreservation success.

5. Conclusion

The addition of AFP I does not affect the survival and viability of *in vivo*-derived sheep embryos cryopreserved. The supplementation of 0.1 μg/mL of AFP I in slow freezing solution enhances mitochondrial activity within 24 h of IVC, maintaining oxidative stress homeostasis and gene modulation, being a potential supplementation to be applied in slow freezing medium of sheep embryos.

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CRediT authorship contribution statement

Lucas F.L. Correia: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Gabriela R. Leal:** Conceptualization, Formal analysis, Writing – original draft. **Felipe Z. Brandão:** Conceptualization, Writing – review & editing. **Ribrio I.T.P. Batista:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. **Joanna M.G. Souza-Fabjan:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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