Cryobiology 95 (2020) 110–115

Contents lists available at ScienceDirect

Cryobiology

journal homepage: http://www.elsevier.com/locate/cryo

Gene expression patterns of *in vivo*-derived sheep blastocysts is more affected by vitrification than slow freezing technique

Viviane L. Brair [a,**], Ana Lucia R.S. Maia [a], Lucas Francisco L. Correia [a], Nathalia O. Barbosa [a], Juliana D.R. Santos [a], Felipe Z. Brandão [a], Jeferson F. Fonseca [b], Ribrio Ivan T.P. Batista [a], Joanna M.G. Souza-Fabjan [a,*]

[a] Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brasil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[b] Embrapa Caprinos e Ovinos, Núcleo Regional Sudeste, Rodovia MG 133, Km 42, CEP 36155-000, Coronel Pacheco, MG, Brazil

ARTICLE INFO

Keywords:
Embryo cryopreservation
Embryonic metabolism
Apoptosis regulator
Implantation
Ovine

ABSTRACT

Transfer of fresh sheep embryos frequently results in higher pregnancy rate compared to cryopreserved ones, possibly due to a failure in the communication between the cryopreserved embryo and the endometrium during pre-implantation and pregnancy establishment. Thus, this study assessed the effect of sheep embryo cryopreservation (slow freezing or vitrification) on embryo survival rate and expression of genes related to trophectoderm differentiation (*CDX2*), pluripotency maintenance (*NANOG*), cell proliferation (*TGFB1*), mitochondrial activity (*NRF1*) and apoptosis (*BAX* and *BCL2*). Superovulation (n = 32 ewes) was performed and embryos were transcervically collected. One hundred good quality (Grade I and II) embryos were allocated into three groups: fresh embryos (CTL; n = 15), slow freezing (SF; n = 42) or vitrification (VT; n = 43). After thawing/warming, three pools of five blastocysts per group were used for RT-qPCR; the remaining 55 embryos were cultured *in vitro* in SOFaa medium at 38.5 °C and 5% CO_2 (SF: n = 27 and VT: n = 28). Survival rate of SF and VT were, respectively, 29.6% (8/27) and 14.2% (4/28) at 24 h; and 48.1% (13/27) and 32.1% (9/28) at 48 h ($P > 0.05$). Only *CDX2* was affected (up-regulated, $P < 0.05$) in both groups compared to CTL. The *BAX* transcript was upregulated in VT, compared to SF group. The VT increased ($P < 0.05$) the expression of all genes, except for *NANOG* and *NRF1*, when compared to the CTL. In conclusion, although *in vitro* survival was similar between techniques, VT led to increased changes in blastocyst gene expression compared to CTL and SF.

1. Introduction

The successful cryopreservation of sheep embryos can improve all other reproductive biotechnologies, such as multiple ovulation and embryo transfer or *in vitro* embryo production. However, lower pregnancy rates after transferring cryopreserved embryos, compared to fresh embryos are reported [13,18,39], regardless of the origin of embryos [either *in vivo*-derived (IVD) or *in vitro* produced (IVP)]. The decrease in the developmental competence of mammalian embryos after cryopreservation is mainly associated with the morphological and functional damage that the cell suffers during the process. The extent of the cryogenic lesion is highly variable and depends on the species, stage of development, embryo origin [11] and cryopreservation technique (slow freezing or vitrification). Slow freezing (SF) is the most widespread cryopreservation technique and its main advantage is the reduced cellular toxicity due to low cryoprotectant concentration; however, it does allow the formation of ice crystals that can lead to cell damage. The opposite can be achieved in the vitrification (VT): no crystallization occurs, due the production of a glassy state of high viscosity to behave like a solid [41], but cryoprotectant toxicity is its major problem. In cattle, both SF and VT resulted in similar cryosurvival and pregnancy rates for IVD embryos [34]. However, in sheep these data are still conflicting.

During embryo development, morphological changes such as cleavages, compaction, blastulation, implantation and gastrulation [51,55] are accompanied by changes in the transcript levels of genes associated with differentiation. These genes are expressed in specific stages and cells, orchestrating the formation of different types of cells, tissues and

* Corresponding author.
** Corresponding author.
E-mail addresses: vivilopesbrair@gmail.com (V.L. Brair), joannavet@gmail.com, jsouza-fabjan@id.uff.br (J.M.G. Souza-Fabjan).

https://doi.org/10.1016/j.cryobiol.2020.05.009
Received 7 April 2020; Received in revised form 19 May 2020; Accepted 21 May 2020
Available online 15 June 2020

organs. Transcription factors required for maintenance of pluripotency, such as POU class 5 homeobox 1 (*POU5F1* or *OCT4*), SRY-box transcription factor 2 (*SOX2*) and Nanog homeobox (*NANOG)*, expressed strictly in cells of the internal cell mass (ICM), are mediated by the action of the caudal type homeobox 2 (*CDX2*) gene product expressed on trophectoderm cells [9,43] have been described in blastocysts.

Mitochondria are responsible for producing the most energy that drives the embryo development and it plays an important role in calcium homeostasis and fatty acid oxidation [33]. The embryo only begins to replicate it in the hatched blastocyst stage, so all previous stages depend on the pre-existing mitochondria in the oocyte [53]. Therefore, only during the implantation period the embryo can replicate mitochondrial DNA [54,59]. The increase in mitochondrial DNA transcription and replication in the blastocyst stage in ruminants is coordinated by the nuclear respiratory factor 1 (*NRF1*) gene [10,35]. Thus, the alteration in this gene expression may compromise mitochondrial proliferation and physiology, leading to impairment in cellular homeostasis. In addition, the anti-apoptotic (B-cell lymphoma protein 2; *BCL2*) and proapoptotic (BCL-2 associated protein X; *BAX*) genes, members of the BCL-2 family, mediate the release of cytochrome C from the intermembrane space of mitochondria, triggering programmed cell death [2]. The relative expression of these genes can be used as a predictor of embryonic competence.

Biopsies derived from IVP blastocysts that resulted in the delivery of calves were enriched with transcripts necessary for implantation, carbohydrate metabolism, growth factor and placentation [16]. An increase in the expression of genes associated with cell survival, growth and proliferation was detected in blastocysts of high cryosurvival [34]. Growth factors, such as transforming growth factor beta 1 (*TGFB1*), involved in cell proliferation and differentiation are also used as markers of embryonic competence [40]. Indeed, the knowledge about the gene expression profile after cryopreservation is crucial for better understanding the mammal embryo development and may be useful to further refinement of embryo cryopreservation techniques. Thus, this study assessed the effect of cryopreservation techniques (SF or VT) on embryo survival rate and expression of genes related to pre-implantation of IVD sheep blastocysts.

2. Material and methods

2.1. Ethics, location and experimental conditions

This research was conducted under the principles of the Brazilian Society of Laboratory Animal Science with approved by the Animal Care Committee of Universidade Federal Fluminense (# 5956101218/2019). The experiment was conducted during April and May (breeding season) of 2019 in Coronel Pacheco (21° 35′ S and 43° 15′ W) in Minas Gerais state, Brazil. All animals underwent gynecological and andrological examinations and had a mean body weight of 57 ± 12 kg and body condition score of 3.5 ± 0.4 (scale 1–5) [58]. They were kept in an intensive system and fed corn silage, supplemented with concentrate provided on demand [36]. Mineralized salt (Salminas Sheep®, Nutriplan, Juiz de Fora, Brazil) and drinking water were available *ad libitum*.

2.2. Experimental design

Ewes (n = 32) were superovulated and embryos were retrieved by non-surgical embryo recovery (NSER), with recovery rate of 65%. A hundred viable embryos [18 compact morulae (Mc), 13 initial blastocysts (Bi), 32 blastocysts (Bl) and 37 expanded blastocysts (Bx)] were allocated into three experimental groups: fresh embryos as SF (n = 42) or VT (n = 43), and blastocysts in control (CTL; n = 15). After cryopreservation, embryos were thawed (SF) or warmed (VT), and then allocated into two trials: 1) Gene expression – three pools of five blastocysts from SF (n = 15: 7 Bx, 6 Bl, 2 Bi), VT (n = 15: 6 Bx, 8 Bl, 1 Bi) and CTL (n = 15: 6 Bx, 3 Bl, 6 Bi) were dry frozen in cryotubes (free of RNase and DNase) at - 196 °C until molecular analysis and three replicates for each pool were performed. The RT-qPCR was made from gene transcripts related to embryo pre-implantation (*CDX2*, *NANOG*, *TGFB1*, *NRF1, BAX*, *BCL2*); 2) *in vitro* culture: embryos from SF (n = 27: 11 Bx, 6 Bl, 1 Bi, 9 Mc) and VT (n = 28: 7 Bx, 9 Bl, 3 Bi, 9 Mc) were cultured in SOFaa medium (BIOK SOF®, Bioklone Reprodução Animal, Jaboticabal, SP, Brazil), at 38.5 °C and 5% CO_2. The survival rate was assessed at 24 and 48 h.

2.3. Embryo recovery and classification

Ewes (n = 32) were synchronized and superovulated as reported by Figueira et al. [18]. Estrus was monitored twice daily, and the ewes were mated by fertile rams (4:1 ratio). Embryos were recovered between the sixth and seventh day of the estrous cycle (D0 = estrus onset), by non-surgical embryo recovery (NSER) after cervical dilation protocol, described previously [21]. All recovered structures were transferred to the holding medium (Holding Plus®, Cultilab, Campinas, Brazil) and classified according to their development/stage (Mc, Bi, Bl, Bx), and quality. Only GI and GII embryos were used [56].

2.4. Cryopreservation of embryos

2.4.1. Slow freezing and thawing

Slow freezing procedures were based on the method previously described [19,20]. Ethylene glycol (EG; 1.5 M) was used in one step with a base solution (BS: 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 in −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; and then plunging into LN_2 for storage. Thawing was performed at room temperature for 5 s, then in a water bath at 36 °C for 30 s.

2.4.2. Vitrification and warming

Vitrification was conducted according to Gibbons et al. [23]. The method was separated in four steps with increasing concentrations of cryoprotectants, and last step, with low time exposure of cryoprotectants with the embryos. 1) BS, for 5 min; 2) BS + 10% glycerol (G) for 5 min; 3) BS + 10% G + 20% EG for 5 min; and 4) BS + 25% G + 25% EG for 30 s. After these steps, the tips with embryos were introduced into 3.6 mL cryotubes filled with LN_2. For warming, the tips were warmed between the thumb and middle finger for 10 s. In the media, sucrose was included for osmolarity control, and each step took 5 min at 25 °C: 1) 12.5% G + 12.5% EG + 0.5 M sucrose; 2) 0.5 M sucrose; 3) 0.25 M sucrose; 4) BS.

2.5. RNA extraction, reverse transcription, and quantitative PCR amplification

Samples were analyzed by quantitative polymerase chain reaction (qPCR) after reverse transcription [5]. Total RNA was extracted from three pools of five blastocysts per group (CTL, SF and VT) using the RNeasyMicro Kit (Qiagen Inc., Valencia, EUA) according to the manufacturer's instructions and treated with DNase for 15 min to prevent DNA contamination. Elution was performed with 14 μL of RNAase free water and the RNA quantification of each pool was performed using 1 μL of sample on a spectrophotometer (Nanodrop 2000, Wilmington, DE, USA). For reverse transcription, using the SuperScript III first-strand synthesis Supermix (Invitrogen, Carlsbad, CA, USA), the same RNA concentration was used for all samples. The reverse transcription reaction was prepared by mixing oligo $(dT)_{20}$ primers, dNTP mixture, Superscript III RT, RNase OUT, $MgCl_2$, RT buffer and RNA sample in a final volume of 20 μL. The mixtures were first incubated at 65 °C for 5 min and then for 50 °C for 50 min. The reaction was terminated at 85 °C for 5 min and then chilled on ice. After that, RNase H was added to the

samples and incubated at 37 °C for 20 min.

Relative quantification was performed in triplicate using real-time polymerase chain reaction (ABI Prism 7300 Sequence Detection Systems, Foster City, CA, USA). Reactions (20 μL 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 (1 μL). Negative controls, comprising the PCR reaction mixture without nucleic acids, were also run with each group of samples. Template cDNAs were denatured at 95 °C for 10 min, and all genes were amplified by 40 cycles of a thermal cycling programmed of 95 °C for 15 s, 55 °C for 15 s and 60 °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 [44] for each reaction. The primer efficiency average was 1.89; 1.91; 1.93; 1.91; 1.91; 1.91; 1.93 and 1.98 to *TGFB1*, *NANOG*, *NRF1*, *CDX2*, *BAX*, *BCL2, GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *H2AFZ* (H2A histone family, member Z), respectively. Relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software [32]. The expression of each target gene was normalized using geometric mean of *GAPDH* and *H2AFZ* values. The stability of the reference genes was calculated according to the methodology described by Pfaffl et al. [42], using the BestKeeper - Excel tool. The values of the Pearson correlation coefficient observed for the *GAPDH* (r2 = 0.774) and *H2AFZ* (r2 = 0.745) genes demonstrate stability ($P < 0.01$) of these reference genes.

2.6. Statistical analysis

The embryo survival data were submitted to Fisher's Exact Test. Construction of graphics was performed in GraphPad Prism 8.0.2. Differences were considered significant at $P < 0.05$. Data are given as the mean ± s.d.

3. Results

3.1. Thawing, warming and in vitro culture

The results of embryonic survival rate after cryopreservation are shown in Table 2. No difference ($P > 0.05$) was observed in survival rate at 24 and 48 h of *in vitro* culture when embryos were subjected to SF or VT. When data were pooled regardless of treatment, the average survival at 24 and 48 h was 21.8 and 40.0%, respectively.

3.2. Gene expression

Gene expression of all genes in the three groups are shown in Fig. 1.

Table 2
In vitro culture survival rate of *in vivo*-derived sheep embryos, cryopreserved by either slow freezing (SF) and vitrification (VT) methods, after thawing and warming, respectively.

Group	Embryo survival rate (%)	
	24 h	48 h
VT[a]	4/28 (14.2)	9/28 (32.1)
SF[a]	8/27 (29.6)	13/27 (48.1)
Total	12/55 (21.8)	22/55 (40.0)

Fisher's Exact Test ($P > 0.05$).

[a] VT group contained n = 28 embryos: seven expanded blastocysts, nine blastocysts, three initial blastocysts and nine compact morulae; SF group contained n = 27 embryos: 11 expanded blastocysts, six blastocysts, one initial blastocyst and nine compact morulae.

Regarding SF, the expression of genes related to apoptosis regulators (pro-apoptotic [*BAX*] and anti-apoptotic [*BCL2*]), pluripotency maintenance (*NANOG*), cell proliferation and differentiation (*TGFB1*) mitochondrial activity (*NRF1*) were not altered ($P > 0.05$) in embryos compared to the CTL. Except for the up-regulated *CDX2* gene (trophectoderm differentiation) ($P < 0.05$). The VT group had an increased ($P < 0.05$) the expression of all genes (*BAX*, *BCL2*, *CDX2* and *TGFB1*, except for *NANOG* and *NRF1*, when compared to CTL. In the comparison between both techniques (SF and VT), only the *BAX* gene was up-regulated ($P < 0.05$) in VT group.

4. Discussion

The global analysis of gene expression shows that VT induces a greater change in the profile of gene expression than SF, when compared to fresh embryos. These data may suggest that IVD embryos are more sensitive to the toxic effect of the high concentration of cryoprotectants than to the harmful effects of ice crystals. However, *in vitro* analyses carried out in the present study demonstrate that the embryos cryopreserved by either SF or VT present similar survival *in vitro*. This data is consistent with a previous report [60], where similar *in vitro* re-expansion rate was obtained when both techniques were compared. The VT technique was developed mainly to improve the survival of IVP embryos, which in general have low cryosurvival when subjected to SF. In comparison with IVD embryos, IVP embryos are characterized by a large accumulation of intracytoplasmic lipids and a high amount of cholesterol and unsaturated fatty acids in the membrane [1,17,45]. These aspects can compromise the success of cryopreservation, as they affect the diffusion and osmosis processes, during freezing/vitrification and thawing/warming. Thus, due to the lower amount of lipids, IVD perhaps have impaired developmental capacity when they are vitrified, possibly due to the rapid diffusion of the cryoprotectant and increased

Table 1
Oligonucleotide primers for RT-qPCR analysis.

Gene symbols	Sequence of primers 5′ to 3′	Annealing temperature (°C)	Amplicon size (bp)	References
TGFB1	F:GGAATTCATGCCGCCCTCGGGGCTGCGG R:GGTCTAGATCAGCTGCACTTGCAGGAGCG	63	390	Juengel et al. [29]
NANOG	F:TTCCCTCCTCCATGGATCTG R:AGGAGTGGTTGCTCCAAGAC	53	501	Sanna et al. [50]
NRF1	F:GCAGGTCCTGTGGGAATG R:CTGGGATAAATGCCCGAAG	61	412	Nau et al. [37]
CDX2	F:GCCACCATGTACGTGAGCTAC R:ACATGGTATCCGCCGTAGTC	60	140	Sakurai et al. [49]
BAX	F:CCTGGGATCTTGAAACTCTCCTT R:CTGAGCCAGGCTGAAATCAAAA	60	566	Chakravarthi et al. [8]
BCL2	F:GCCGAGTGAGCAGGAAGAC R:GTTAGCCAGTGCTTGCTGAGA	60	214	Chakravarthi et al. [8]
GAPDH	F:ATGTTTGTGATGGGCGTGAA R:ACAGTCTTCTGGGTGGCAGT	60	176	O'Connor et al. [38]
H2AFZ	F:GTCGTGGCAAGCAAGGAG R:GATCTCGGCCGTTAGGTACTC	57	182	O'Connor et al. [38]

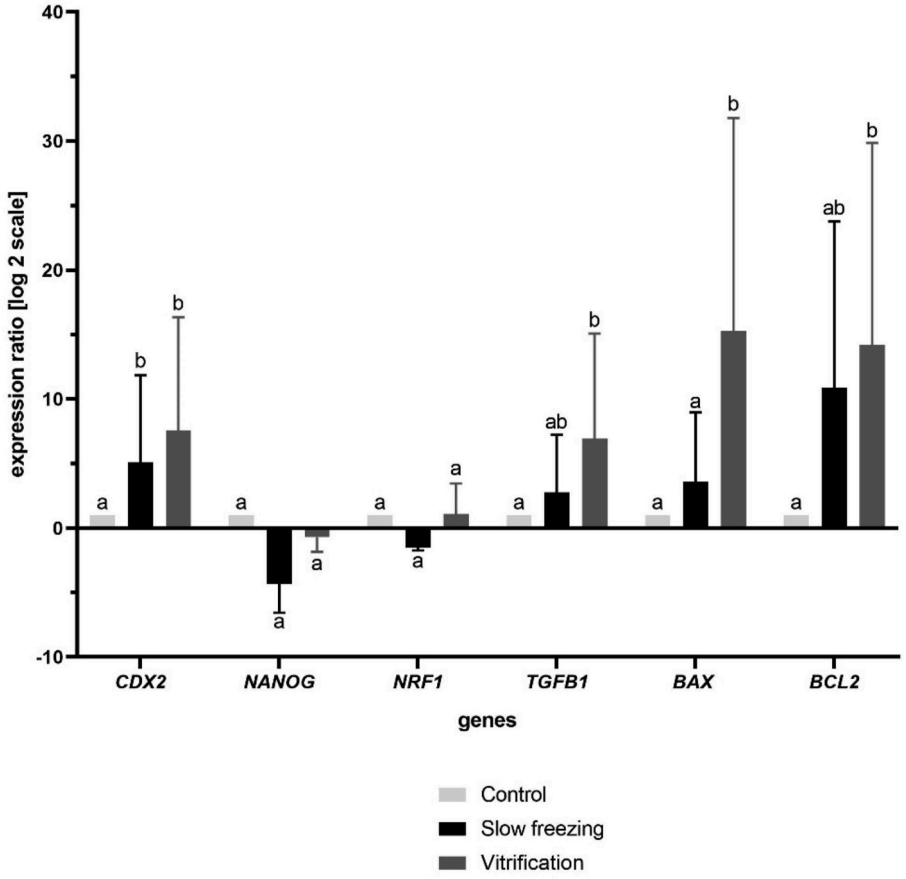

Fig. 1. Gene expression related to trophectoderm differentiation (*CDX2*), pluripotency maintenance (*NANOG*), cell proliferation (*TGFB1*), mitochondrial activity (*NRF1*) and apoptosis (*BAX* and *BCL2*) of fresh sheep blastocysts (Control), and immediately after vitrification/warming or frozen/thawing of blastocysts. Different letters show statistical difference ($P < 0.05$).

relative exposure time to the cryoprotectant.

Mammalian embryos are particularly sensitive to thermal shock [47,48]. The thermal stress may result in homeostatic regulator production as chaperones [15], apoptosis related proteins [61] and expression of genes associated with development capacity as *CDX2* [52]. The balance between pro and anti-apoptotic family members partially determines sensitivity of cell to apoptosis. Anti-apoptotic genes as *BCL2* interact with pro-apoptotic *BAX* genes to counteract their activity. Activation of BAX proteins lead to a breakdown in outer mitochondrial membrane permeability, the release of cytochrome C in cytoplasm, and the activation of caspases responsible for cell death. In the present study, we observed higher expression of the *BAX* gene in embryos from VT, compared to CTL and SF. These data indicate a greater pro-apoptotic stimulus in VT embryos compared to the other groups.

Regardless of cryopreservation technique (SF or VT), *CDX2* gene expression was up-regulated compared to fresh embryos, but this result could hypothetically be affected by the slightly different developmental stages of embryos in the three groups. Studies in cattle and sheep have shown that *IFNT* expression is *CDX2*-dependent [49], which is expressed in blastocysts [6,14,27] and *IFNT* is important signaling the process of maternal recognition of pregnancy [24,26,46]. Increased expression of this gene in cryopreserved embryos (SF and VT) may be a strategy to boost IFN-τ production, since secretion of this protein is compromised in cattle cryopreserved embryos [3]. Supporting this hypothesis, we also observed an increase in *TGFB1* expression in VT-embryos compared to the CTL. The TGF-β1 is a polypeptide member of the TGF-β superfamily of cytokines. This protein when secreted stimulates cell proliferation and differentiation [4,25,30,57]. It is reasonable to assume that its increased expression may be a compensatory mechanism to prevent embryonic death.

In the present study, regardless of the technique, embryo cryopreservation did not affect the expression of *NANOG*, suggesting that cellular stress during cryopreservation does not compromise the ability of embryonic pluripotency to be maintained [9]. Similarly, *NRF1* expression was also unaffected by cryopreservation. These data suggest the need for increased expression of the *NRF1* gene to supply the cell energy production capacity. However, analysis of this gene immediately after cryopreservation demonstrates that its expression is unaffected by ultrastructural and cytotoxic damage, which sheep embryos suffer during cryopreservation [12].

Although *in vitro* culture analysis was similar between groups, the VT group had a significant increase in the expression of all genes, except for *NANOG* and *NRF1*. We believe that the increase of pro-apoptotic gene (*BAX*) found in VT compared to SF, occurred due to high concentration of cryoprotectants used in this technique, stimulating response of cellular stress, due to chemical toxicity or osmotic on cells, beyond to cold stress of cryopreservation [41]. Leoni et al. [31] evaluated genes related to the water movement (*AQP3*: Aquaporin 3/*ATP1A1*: ATPase Na^+/K^+ transporting subunit alpha 1) of IVP sheep blastocyst after VT. After warming, embryos were cultured for 8 h and 16 h, and the gene expression was evaluated on re-expanded blastocysts. The authors observed the decrease of *ATP1A1* and increase of *AQP3* from 8 h to 16 h, being inversely proportional. In addition, Iwayama et al. [28] and Frank et al. [22] suggested that VT technique compromises the primary mechanism of water movement by *ATP1A1*, activating a second movement mechanism by *AQP3*. However, *AQP3* is known for its non-exclusive water permeability, being also permeable to other small solutes and glycerol [7]. In our study, glycerol was used in the VT

protocol, resulting in greater intracellular toxicity, response to cell stress and apoptosis than in SF, possibly due to the increase in cryoprotectant influx by *AQP3*. This corroborates with the up-regulation of the *BAX* pro-apoptotic gene founded in vitrified IVD sheep embryos.

In conclusion, *in vivo*-derived embryos submitted to either SF or VT have similar ability to survive *in vitro* but VT led to increased changes in blastocyst gene expression compared to CTL and SF.

Acknowledgements

The authors thank EMBRAPA (Brazilian Agricultural Research Corporation) (Project 22.13.06.026.00.05), CNPq (National Council for Scientific and Technological Development) (Project 434302/2018–0) and FAPERJ (Carlos Chagas Filho Foundation for Support to Research in the State of Rio de Janeiro) for financial support. VLB, ALRSM and JMGS-F are FAPERJ fellows and JFF, JMGS-F and FZB are CNPq fellows.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2020.05.009.

References

[1] H. Abe, S. Yamashita, T. Satoh, H. Hoshi, Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media, Mol. Reprod. Dev. 61 (2002) 57–66.
[2] J.M. Adams, S. Cory, The Bcl-2 apoptotic switch in cancer development and therapy, Oncogene 26 (2007) 1324–1337.
[3] M.C.C. Araújo, V.R. Vale Filho, A.M. Ferreira, W.F. Sá, J.B. Barreto Filho, L.S. A. Camargo, R.V. Serapião, M.V.G. B Silva, Interferon tau secretion in cattle embryos *in vitro* fertilized before and after cryopreservation, Arq. Bras. Med. Vet. Zootec. 57 (2005) 751–756.
[4] A. Barrera, E. García, D. Miceli, Effect of exogenous transforming growth factor β1 (*TGF-β1*) on early bovine embryo development, Zygote 26 (2018) 232–241.
[5] R.I.T.P. Batista, M.C.S. Luciano, D.I.A. Teixeira, V.J.F. Freitas, L.M. Melo, Methodological strategies for transgene copy number quantification in goats (*Capra hircus*) using Real-Time PCR, Biotechnol. Prog. 30 (2014) 1390–1400.
[6] D.K. Berg, C.S. Smith, D.J. Pearton, D.N. Wells, R. Broadhurst, M. Donnison, P. L. Pfeffer, Trophectoderm lineage determination in cattle, Dev. Cell 20 (2011) 244–255.
[7] M. Borgnia, S. Nielsen, A. Engel, P. Agre, Cellular and molecular biology of the aquaporin water channels, Annu. Rev. Biochem. 68 (1999) 425–458.
[8] V.P. Chakravarthi, S.S. Kona, A.V.S. Kumar, M. Bhaskarand, V.H. Rao, Quantitative expression of antiapoptotic and proapoptotic genes in sheep ovarian follicles grown *in vivo* or cultured *in vitro*, Theriogenology 83 (2015) 590–595.
[9] I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie, A. Smith, Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells, Cell 113 (2003) 643–655.
[10] M.R. Chiaratti, C.R. Ferreira, F. Perecin, S.C. Meo, J.R. Sangalli, L.G. Mesquita, J. C. Carvalho Balieiro, L.C. Smith, J.M. Garcia, F.V. Meirelles, Ooplast-mediated developmental rescue of bovine oocytes exposed to ethidium bromide, Reprod. Biomed. Online 22 (2011) 172–183.
[11] L. Dalcin, C.M. Lucci, Cryopreservation of livestock embryos: cryobiological principles and current status, Rev. Bras. Reprod. Anim. 34 (2010) 149–159.
[12] L. Dalcin, R.C. Silva, F. Paulini, B.D.M. Silva, J.P. Neves, C.M. Lucci, Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos, Cryobiology 67 (2013) 137–145.
[13] M. Dattena, G. Ptak, P. Loi, P. Cappair, Survival and viability of vitrified *in vivo* and *in vitro* produced ovine blastocysts, Theriogenology 53 (2000) 1511–1519.
[14] S.A. Degrelle, E. Campion, C. Cabau, F. Piumi, P. Reinaud, C. Richard, J.-P. Renard, I. Huea, Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts, Dev. Biol. 288 (2005), 448-46.
[15] J.L. Edwards, P.J. Hansen, Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocytes, Biol. Reprod. 55 (1996) 341–346.
[16] A. El-Sayed, M. Hoelker, F. Rings, D. Salilew, D. Jennen, E. Tholen, M.A. Sirard, K. Schellander, D. Tesfaye, Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients, Physiol. Genom. 28 (2006) 84–96.
[17] T. Fair, P. Lonergan, A. Dinnyes, D.C. Cottell, P. Hyttel, F.A. Ward, M.P. Boland, Ultrastructure of bovine blastocysts following cryopreservation: effect of method of blastocyst production, Mol. Reprod. Dev. 58 (2001) 186–195.
[18] L.M. Figueira, N.G. Alves, J.M.G. Souza-Fabjan, R.I.T.P. Batista, L.C. Souza, A.L.R. S. Maia, V.L. Brair, M. Filgueiras, G.N. Souza, J.F. Fonseca, Superovulation and transcervical embryo recovery in Lacaune ewes raised under tropical conditions, Anim. Reprod. 15 (2018) 402. Annual Meeting of the Brazilian Embryo Technology Society (SBTE).
[19] L.M. Figueira, N.G. Alves, R.I.T.P. Batista, V.L. Brair, R.R. Lima, M.E.F. Oliveira, J. F. Fonseca, J.M.G. Souza-Fabjan, Pregnancy rate after fixed-time transfer of cryopreserved embryos collected by non-surgical route in Lacaune sheep, Reprod. Domest. Anim. 54 (2019) 1493–1496.
[20] J.F. Fonseca, R.I.T.P. Batista, J.M.G. Souza-Fabjan, M.E.F. Oliveira, F.Z. Brandão, J. H.M. Viana, Freezing goat embryos at different developmental stages and quality using ethylene glycol and a slow cooling rate, Arq. Bras. Med. Vet. Zootec. 70 (2018) 1489–1496.
[21] J.F. Fonseca, F.N. Zambrini, J.D. Guimarães, M.R. Silva, M.E.F. Oliveira, F. Z. Brandão, P.M. Bartlewski, J.M.G. Souza-Fabjan, Combined treatment with estradiol benzoate, d-cloprostenol and oxytocin permits cervical dilation and non-surgical embryo recovery in ewes, Reprod, Domest. Anim. 54 (2018) 118–125.
[22] L.A. Frank, R.D. Rose, M.R. Anastasi, T.C.Y. Tan, M.F. Barry, J.G. Thompson, H. M. Brown, Artificial blastocyst collapse prior to vitrification significantly improves Na+/K+-ATPase-dependent post-warming blastocoel re-expansion kinetics without inducing endoplasmic reticulum stress gene expression in the mouse, Reprod. Fertil. Dev. 31 (2018) 294–305.
[23] A. Gibbons, M.I. Cueto, F. Pereyra Bonnet, A simple vitrification technique for sheep and goat embryo cryopreservation, Small Rumin. Res. 95 (2011) 61–64.
[24] J.D. Godkin, F.W. Bazer, J. Moffatt, F. Sessions, R.M. Roberts, Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at Day 13–21, Reproduction 65 (1982) 141–150.
[25] P. HosseinNia, M. Tahmoorespur, S.M. Hosseini, M. Hajian, S. Ostadhosseini, M. R. Nasiri, M.H. Nasr-Esfahani, Stage-specific profiling of Transforming Growth Factor-β, fibroblast growth factor and wingless-int signaling pathways during early embryo development in the goat, Cell J 17 (2016) 648–658.
[26] K. Imakawa, R.V. Anthony, M. Kazemi, K.R. Marotti, H.G. Polites, R.M. Roberts, Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm, Nature 330 (1987) 377–379.
[27] K. Imakawa, M. Kim, F. Matsuda-Minehata, S. Ishida, M. Iizuka, M. Suzuki, K. Chang, S.E. Echternkamp, R.K. Christenson, Regulation of the ovine interferon-tau gene by a blastocyst-specific transcription factor, CDX2,, Mol. Reprod. Dev. 73 (2006) 559–567.
[28] H. Iwayama, S. Hochi, M. Yamashita, *In vitro* and *in vivo* viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification, J. Assist. Reprod. Genet. 28 (2011) 355–361.
[29] J.L. Juengel, A.H. Bibby, K.L. Reader, S. Lun, L.D. Quirke, L.J. Haydon, K. P. McNatty, The role of transforming growth factor-beta (TGF-beta) during ovarian follicular development in sheep, Reprod. Biol. Endocrinol. 78 (2004) 1–11.
[30] W.A. Kues, S. Sudheer, D. Herrmann, J.W. Carnwath, V. Havlicek, U. Besenfelder, H. Lehrach, J. Adjaye, H. Niemann, Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development *in vivo*, P.N.A.S. 105 (2008) 19768–19773.
[31] G.G. Leoni, F. Berlinguer, S. Succu, D. Bebbere, F. Mossa, M. Madeddu, S. Ledda, L. Bogliolo, S. Naitana, A new selection criterion to assess good quality ovine blastocysts after vitrification and to predict their transfer into recipients, Mol. Reprod. Dev. 75 (2008) 373–382.
[32] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method, Methods 25 (2001) 402–408.
[33] B. Lledo, J.A. Ortiz, R. Morales, E. García-Hernández, J. Ten, A. Bernabeu, J. Llácer, R. Bernabeu, Comprehensive mitochondrial DNA analysis and IVF outcome, Hum. Reprod. Open 4 (2018) 1–9.
[34] T.V. Marsico, J. Camargo, R.S. Valente, M.J. Sudano, Embryo competence and cryosurvival: molecular and cellular features, Anim. Reprod. 16 (2019) 423–439.
[35] P. May-Panloup, M.F. Chretien, C. Jacques, C. Vasseur, Y. Malthiery, P. Reynier, Low oocyte mitochondrial DNA content in ovarian insufficiency, Hum. Reprod. 20 (2005) 593–597.
[36] National Research Council - NRC, Nutrient Requirements of Goats, The National Academies Press, Washington, D.C.), 2007, 2007.
[37] P.N. Nau, T.V. Natta, J.C. Ralphe, C.J. Teneyck, K.A. Bedell, C.A. Caldarone, J. L. Segar, T.D. Scholz, Metabolic adaptation of the fetal and postnatal ovine heart: regulatory role of hypoxia-inducible factors and nuclear respiratory factor-1, Pediatr. Res. 52 (2002) 269–278.
[38] T. O'Connor, I. Wilmut, J. Taylor, Quantitative evaluation of reference genes for real-time PCR during *in vitro* maturation of ovine oocytes, Reprod. Domest. Anim. 48 (2013) 477–483.
[39] S. Papadopoulos, D. Rizos, P. Duffy, M. Wade, K. Quinn, M.P. Boland, P. Lonergan, Embryo survival and recipient pregnancy rates after transfer of fresh or vitrified, *in vivo* or *in vitro* produced ovine blastocysts, Anim. Reprod. Sci. 74 (2002) 35–44.
[40] B.C. Paria, K.L. Jones, K.C. Flanders, S.K. Dey, Localization and binding of transforming growth factor-β isoforms in mouse preimplantation embryos and in delayed and activated blastocysts, Dev. Biol. 151 (1992) 91–104.
[41] D.E. Pegg, Principles of cryopreservation, Methods Mol. Biol. 1257 (2015) 3–19.
[42] M.W. Pfaffl, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: best Keeper-Excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509–515.
[43] A. Ralston, J. Rossant, Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo, Dev. Biol. 313 (2008) 614–629.
[44] C. Ramakers, J.M. Ruijter, R.H.L. Deprez, A.F.M. Moorman, Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, Neurosci. Lett. 339 (2003) 62–66.
[45] D. Rizos, F. Ward, P. Duffy, M.P. Boland, P. Lonergan, Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*:

implications for blastocyst yield and blastocyst quality, Mol. Reprod. Dev. 61 (2002) 234–248.
[46] R.M. Roberts, D.W. Leaman, J.C. Cross, Role of interferons in maternal recognition of pregnancy in ruminants, Exp. Biol. Med. 200 (1992) 7–18.
[47] R. Romão, E. Bettencourt, R.M.L.N. Pereira, C.C. Marques, M.C. Baptista, J. P. Barbas, E. Oliveira, C. Bettencourt, M. Sousa, Ultrastructural characterization of fresh and vitrified *in vitro* and *in vivo* produced sheep embryos, Anat. Histol. Embryol. 45 (2015) 231–239.
[48] M. Sakatani, L. Bonilla, K.B. Dobbs, J. Block, M. Ozawa, S. Shanker, J. Yao, P. J. Hansen, Changes in the transcriptome of morula-stage bovine embryos caused by heat shock: relationship to developmental acquisition of thermotolerance, Reprod. Biol. Endocrinol. 11 (2013) 3.
[49] T. Sakurai, H. Bai, T. Konno, A. Ideta, Y. Aoyagi, J.D. Godkin, K. Imakawa, Function of a transcription factor cdx2 beyond its trophectoderm lineage specification, Endocrinology 151 (2010) 5873–5881.
[50] D. Sanna, A. Sanna, L. Mara, S. Pilichi, A. Mastinu, F. Chessa, L. Pani, M. Dattena, Oct4 expression in in-vitro-produced sheep blastocysts and embryonic-stem-like cells, Cell Biol. Int. 34 (2010) 53–60.
[51] N. Schrode, P. Xenopoulos, A. Piliszek, S. Frankenberg, B. Plusa, A. K. Hadjantonakis, Anatomy of a blastocyst: cell behaviors driving cell fate choice and morphogenesis in the early mouse embryo, Genesis 51 (2013) 219–233.
[52] C.F. Silva, E.S. Sartorelli, A.C.S. Castilho, R.A. Satrapa, R.Z. Puelkera, E.M. Razza, J.S. Ticianelli, H.P. Eduardo, B. Loureiro, C.M. Barros, Effects of heat stress on development, quality and survival of Bos indicus and Bos taurus embryos produced *in vitro*, Theriogenology 79 (2013) 351–357.
[53] E.C. Spikings, J. Alderson, J.C. St John, Transmission of mitochondrial DNA following assisted reproduction and nuclear transfer, Hum. Reprod. Update 12 (2006) 401–415.
[54] J.C. St John, J. Facucho-Oliveira, Y. Jiang, R. Kelly, R. Salah, Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells, Hum. Reprod. Update 16 (2010) 488–509.
[55] R.O. Stephenson, J. Rossant, P.P.L. Tam, Intercellular interactions, position, and polarity in establishing blastocyst cell lineages and embryonic axes, CSH Perspect. Biol. 4 (2012) a008235.
[56] D.A. Stringfellow, M.D. Givens, Manual of the International Embryo Transfer Society, fourth ed., International Embryo Transfer Society, Champaign, 2010.
[57] S. Sudheer, J. Adjaye, Functional genomics of human pre-implantation development, Brief. Funct. Genomic 6 (2007) 120–132.
[58] J. Suiter, Body Condition Scoring in Sheep and Goats, Farmnote (1994) 69–94.
[59] G.A. Thouas, A.O. Trounson, G.M. Jones, Effect of female age on mouse oocyte developmental competence following mitochondrial injury, Biol. Reprod. 73 (2005) 366–373.
[60] F.C. Varago, V.S. Moutacas, B.C. Carvalho, R.V. Serapião, F. Vieira, H. Chiarini-Garcia, F.Z. Brandão, L.S. Camargo, M. Henry, M.A. Lagares, Comparison of conventional freezing and vitrification with dimethylformamide and ethylene glycol for cryopreservation of ovine embryos, Reprod. Domest. Anim. 49 (2014) 839–844.
[61] R.J. Youle, A. Strasser, The BCL-2 protein family: opposing activities that mediate cell death, Nat. Vet. Mol. Cell. Biol. 9 (2008) 47–59.