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SHORT COMMUNICATION

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Cryoprotectant role of exopolysaccharide ID1 in the vitrification/in-straw warming of in vitro-produced bovine embryos

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Abstract

The cold-adapted bacterium *Pseudomonas sp.* ID1 produces the extracellular exopolysaccharide ID1 (EPS ID1) with cryoprotective activity. This study was designed to optimize the vitrification/in-straw warming protocol of *in vitro*-produced (IVP) blastocysts by adding EPS ID1 to the vitrification media. Day 7-expanded blastocysts were vitrified/warmed using the VitTrans device after the addition of 0 or 100 μg/mL EPS ID1 to the vitrification media. Blastocysts vitrified by the Cryotop method and fresh non-vitrified blastocysts served as controls. Outcomes were assessed in the warmed embryos in terms of survival rates and mRNA relative abundances of *BAX*, *BCL2*, *GPX1*, and *CDX2* genes. No differences in survival rates were observed at 3 h post-warming between vitrification treatments. At 24 h post-warming, the addition of EPS prior to vitrification with the VitTrans device produced similar survival rates to Cryotop-vitrified embryos and similar hatching rates to fresh non-vitrified or Cryotop-vitrified embryos. No differences emerged in *BCL2* gene expression. Lower *BAX* ($p < .05$) and higher *GPX1* ($p < .05$) and *CDX2* ($p < .1$) gene expression were observed in expanded and/or hatched blastocysts derived from VitTrans-EPS-vitrified embryos when compared to those from the non-supplemented group. In conclusion, addition of EPS not only promoted blastocyst survival and hatching after VitTrans vitrification/warming but also modified the expression of genes associated with better embryo quality.

KEYWORDS

apoptosis, blastocyst, cell differentiation, cryopreservation, gene expression, oxidative stress, survival rate

1 | INTRODUCTION

Cryopreservation of in vitro-produced (IVP) bovine embryos is a critical step to ensure the widespread reproduction and conservation of high-value animals. Vitrification appears to be the most efficient approach for IVP embryos, which are more sensitive to cryoinjury than their in vivo counterparts (Rizos et al., 2001). Vitrification, however, requires a stereomicroscope during the stepwise warming procedure and trained personnel to examine embryos before transfer, limiting its application on a large scale. VitTrans is a device that allows field-warming/dilution and embryo transfer directly to female recipients. Vitrification of IVP day 7 bovine blastocysts using the VitTrans vitrification/warming device and short exposure to the CPA equilibration solution results in post-warming outcomes comparable to those of fresh non-vitrified blastocysts (Martinez-Rodero et al., 2021).

Under hostile marine conditions, microorganisms produce several secondary metabolites and exopolysaccharides (EPS) as a part of their survival strategy. Exopolysaccharide ID1 (EPS ID1) is produced by *Pseudomonas sp.*, a cold-adapted bacterium isolated from marine sediments in Antarctica. Not only does EPS ID1 cryoprotective activity benefit the cold-adapted bacterial producer, but also non-producing cells (Carillo et al., 2015). Arcarons et al. (2019) found that the addition of EPS ID1 to the vitrification/warming media confers significant cryoprotection to in vitro matured bovine oocytes, by preserving spindle/chromosome dynamics and improving embryonic developmental competence.

This study aimed to optimize vitrification and in-straw warming of bovine IVP embryos by adding EPS ID1 to the vitrification solutions. Outcomes were assessed in the warmed embryos in terms of survival rates, and relative abundances of mRNAs of genes with a role in apoptosis, oxidative stress, and cell differentiation.

2 | MATERIALS AND METHODS

2.1 | In vitro embryo production

Procedures for in vitro maturation, in vitro fertilization, and in vitro culture are thoroughly described elsewhere (Martinez-Rodero et al., 2021).

2.2 | Embryo vitrification and warming

Day 7-expanded blastocysts were randomly allocated into three groups: (1) Cryotop, blastocysts were vitrified/warmed following the short equilibration protocol of the Cryotop method (Rizos et al., 2001; Walton et al., 2017); (2) VitTrans, blastocysts were vitrified/warmed following the short equilibration VitTrans protocol described in Martinez-Rodero et al. (Martinez-Rodero et al., 2021) (Figure 1); (3) VitTrans-EPS ID1, blastocysts were vitrified/warmed by the VitTans protocol but vitrification media were supplemented with 100 µg/mL EPS ID1 as already described in Ordóñez-León et al. (2022). Non-vitrified blastocysts served as the fresh control. After warming, blastocysts were transferred to SOF culture medium and incubated at 38.5°C in a 5% CO_2 and 5% O_2 humidified atmosphere. Survival rates were expressed as rates of re-expanded blastocysts at 3 h and 24 h post-warming. Hatching rates were defined as the proportions of hatching/hatched blastocysts at 24 h post-warming. Five independent experiments were conducted.

2.3 | RNA extraction, reverse transcription, and quantitative Real-Time PCR analysis

Surviving vitrified/warmed blastocysts at 24 h post-warming were classified as expanded or hatching blastocysts, pooled up to 5 blastocysts, snap-frozen in liquid nitrogen, and kept at −80°C until RNA isolation and RT-qPCR analysis were performed. Total RNA was extracted using RNeasy Kit (Qiagen,) following the manufacturer's instructions. RNA concentration and quality were determined using the Epoch spectrophotometer (BioTek,). The resulting RNA was reverse transcribed according to the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems,) instructions. The qPCR was performed using a 7500 Real Time PCR System (Applied Biosystems,), and a reaction mixture consisting of 10 µl of Fast SYBR Green Master Mix (Thermo Fisher,), 1.2 µl of each primer (300 nM; Thermo Fisher; Table 1), and 2 µl of the cDNA template. The relative abundance of four target genes (*BAX*, *BCL2*, *GPX1*, and *CDX2)* was measured by the Livak method (Livak & Schmittgen, 2001), using the *PPIA* housekeeping gene as normalizer. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta Ct)}$. Calculation of ΔΔCt involved the subtraction of the ΔCt value for the fresh embryo control group from all the other ΔCt sample values. The experiment was repeated independently five times.

2.4 | Statistical analysis

The software IBM SPSS Version 25.0 (IBM Corp.,) was used to perform all statistical tests. Normal distribution and homogeneity of variances were checked with Shapiro–Wilk and Levene tests, respectively. Survival and hatching rates were compared by ANOVA and Bonferroni tests. The relative abundance of genes was analysed by Kruskal–Wallis, and Mann–Whitney tests. The level of statistical significance was set at $p \le .05$.

3 | RESULTS

Vitrification by the VitTrans method led to a significant reduction in embryo survival rates recorded at 3 h post-warming when compared to fresh control blastocysts, regardless of the EPS ID1 treatment. At 24 h post-warming, addition of EPS ID1 to the media prior to VitTrans vitrification of bovine blastocysts produced equivalent embryo survival than when blastocysts were vitrified by the Cryotop method. Hatching rates after VitTrans-EPS ID1 vitrification were similar to those for fresh control and Cryotop-vitrified blastocysts (Table 2).

The levels of BAX gene expression of apoptosis-related genes BAX and were higher in expanded blastocysts derived from VitTrans embryos than in those derived from the Cryotop or VitTrans-EPS ID1 group (Figure 2). Neither the level of expression of the apoptosis-related gene *BCL2* nor the *BAX*/*BCL2* ratio differed significantly

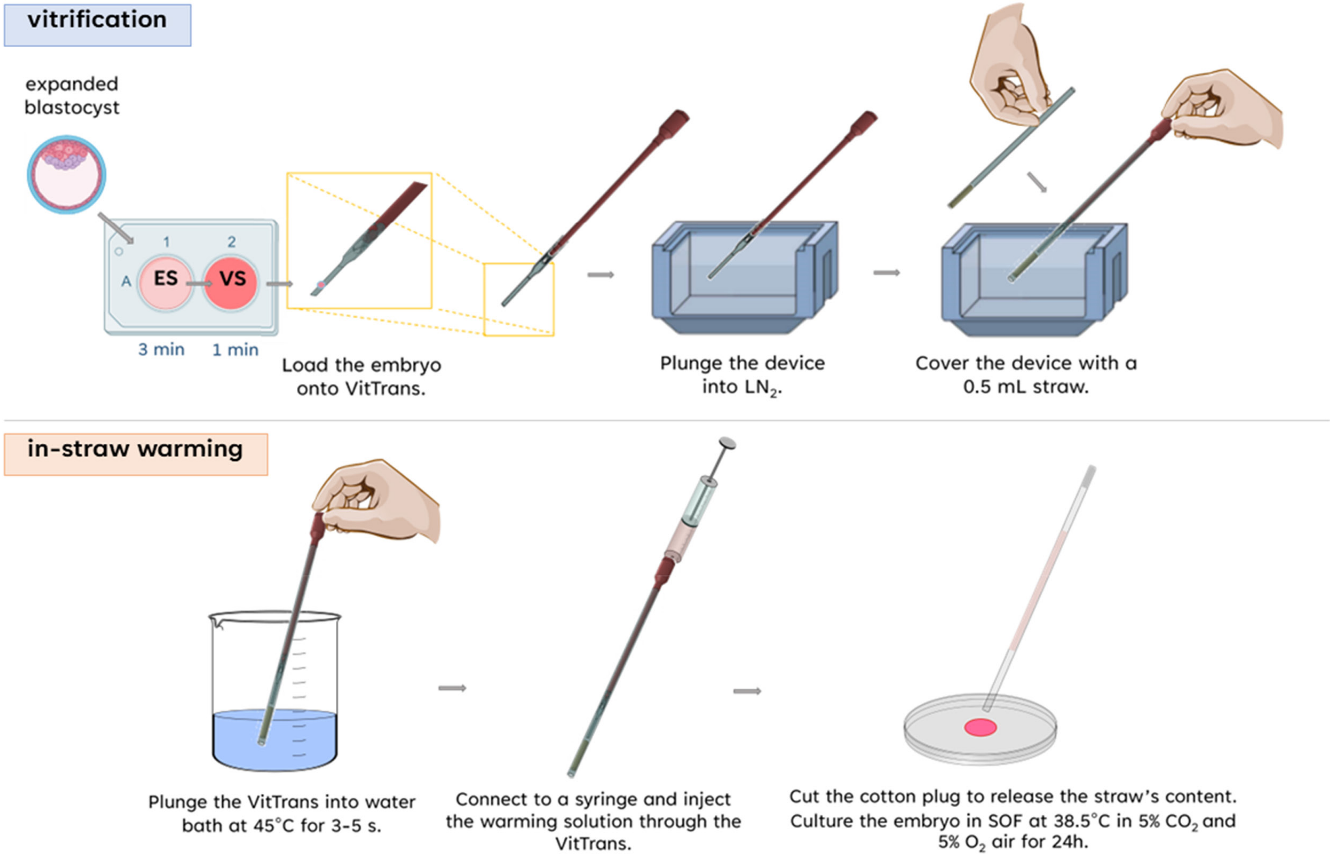

FIGURE 1 Graphical description of VitTrans vitrification/in-straw warming method. The device comprises a carrier where the embryo is loaded, a hard plastic handle with an inner channel into which warming solution is introduced to dilute the cryoprotectant and transport the embryo to the straw for transfer, and a Luer syringe connector to connect the device to the warming solution source. The straw acts as a cover to protect the device from mechanical damage during storage. During warming, it serves as a 0.5 mL straw for sample dilution and direct embryo transfer

TABLE 1 Primers used for reverse transcription-quantitative polymerase chain reaction

Symbol	Primer sequences (5′-3′)	Amplicon size (bp)	GenBank accession *n*°
BAX	Fw: ACCAAGAAGCTGAGCGAGTG Rv: CGGAAAAAGACCTCTCGGGG	116	NM_173894.1
BCL2	Fw: GGCCCCTGTTTGATTTCTCCT Rv: ACTTATGGCCCAGATAGGCAC	99	NM_001166486.1
GPX1	Fw: CTGAAGTACGTCCGACCAGG Rv: GTCGGTCATGAGAGCAGTGG	153	NM_174076.3
CDX2	Fw: TGGACTCTGCTAGAACCCTCA Rv: TTTGTTTCTGCTCGGAGGGC	89	NM_001206299.1
PPIA	Fw: CATACAGGTCCTGGCATCTTGTCC Rv: CACGTGCTTGCCATCCAACC	108	NM_178320.2

between vitrification groups and the control fresh group (Figure 2). Surviving hatched blastocysts derived from the VitTrans-EPS ID1 group, however, displayed significantly higher *GPX1* expression than those derived from hatched blastocysts vitrified using the Cryotop or VitTrans methods. Although not significant, a trend ($p = .06$) to upregulation of the *CDX2* gene was identified in both expanded and hatched blastocysts derived from the VitTrans-EPS ID1 group when compared to blastocysts vitrified using the Cryotop or VitTrans method.

4 | DISCUSSION

This study aimed to investigate how adding EPS ID1 to the vitrification media affects post-warming outcomes in bovine D7 expanded blastocysts vitrified using the vitrification/in straw-warming VitTrans technique. Non-EPS ID1 supplementation resulted in significantly lower survival and hatching rates, whereas the addition of EPS ID1 prior to VitTrans vitrification produced similar survival rates than those observed in embryos vitrified/

TABLE 2 Effects of adding EPS ID1 to the VitTrans vitrification solutions on post-warming survival and hatching rates

Treatment	n	Post-warming Survival (%) (3 h)	Survival (%) (24 h)	Hatching rate (%) (24 h)
Fresh	52	100[a]	100[a]	32.40 ± 3.96[a]
Cryotop	51	50.88 ± 6.80[b]	79.95 ± 3.52[b]	30.66 ± 5.51[a]
VitTrans	63	47.71 ± 5.26[b]	67.45 ± 2.33[c]	14.09 ± 2.60[b]
VitTrans-EPS ID1	67	51.90 ± 5.18[b]	78.98 ± 0.43[b]	32.00 ± 4.79[a]

[a,b]Values within columns with different superscripts differ significantly ($p < .05$); Data are shown as mean ± standard error of the mean (SEM).

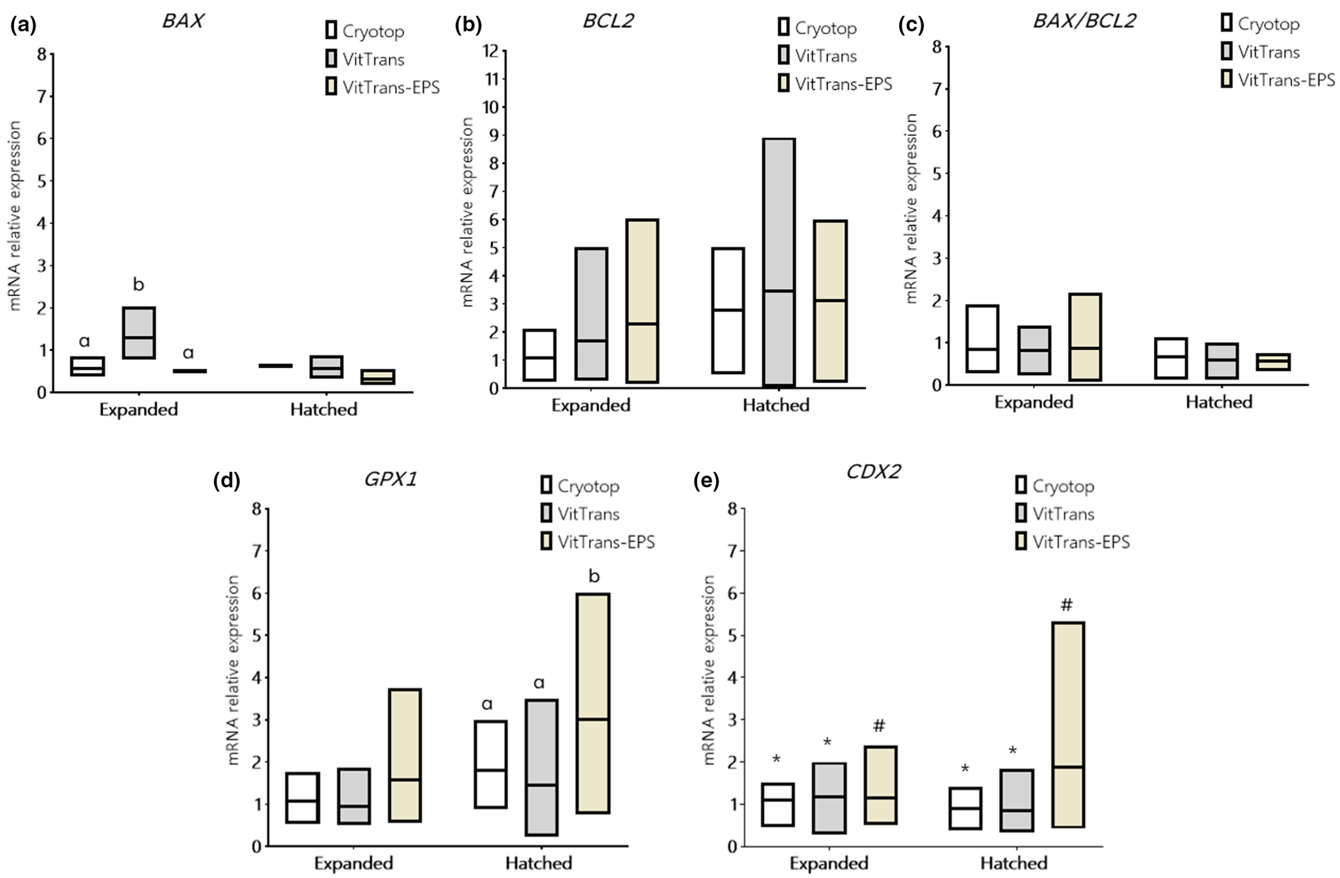

FIGURE 2 Box plots showing relative post-warming gene expression levels of (a) *BAX*, (b) *BCL-2*, (c) *BAX/BCL-2 ratio*, (d) *GPX1*, (e) *CDX2* in bovine expanded and hatched blastocysts derived from blastocysts vitrified/warmed using Cryotop, VitTrans or VitTrans-EPS ID1 protocols. The solid line indicates the mean, and floating bars represent minimum to maximum values. [a,b]Bars labelled with diferent letters indicate a significant difference between treatments within blastocyst stage ($p < .05$). [*,#]Bars labelled with different symbols indicate a trend towards a significant difference between treatments within blastocyst stages ($p < .1$ and $> .05$). *BAX*, BCL-2 associated X apoptosis regulator; *BCL-2*, BCL-2 apoptosis regulator; *GPX1*, glutathione peroxidase 1; *CDX2*, caudal type homeobox 2

warmed by the Cryotop methodology and similar hatching rates than those of fresh non-vitrified embryos. When compared to previous studies where the VitTrans method was used to vitrify IVP bovine embryos, the addition of EPS ID1 to the vitrification media resulted in greater post-warming outcomes (González et al., 2019; Morató & Mogas, 2014). It has already been established that adding EPS ID1 to the vitrification media of in vitro matured bovine oocytes has positive benefits (Arcarons et al., 2019). Although the specific composition and antifreeze properties of EPS ID1 have been described (Carrión et al., 2015), it is difficult to understand how this EPS ID1 provides cryoprotection to cells due to a lack of knowledge about its exact primary and secondary structure. Because EPS ID1 is characterized by amino acids decoration, and amino acids have been shown to be important for ice interactions in EPSs (Carillo et al. 2015), we could hypothesize that EPS ID1 confers cryoprotection by limiting ice recrystallization. To corroborate this hypothesis, more information about the molecular primary and secondary structure of EPS ID1 is required.

Increased *BAX* expression in expanded blastocysts derived from embryos vitrified without EPS may be related to poor quality or fragmented embryos (Yang & Rajamahendran, 2002). Addition of EPS, on the other hand, restored *BAX* levels to those of the Cryotop group. Higher *GPX1* expression in hatching/hatched embryos derived from the VitTrans-EPS ID1 is linked to better embryo quality (Cebrian-Serrano et al., 2013), whereas a tendency of a higher *CDX2* expression in embryos from the VitTrans-EPS ID1 group is associated with improved pregnancy rates after embryo transfer (El-Sayed et al., 2006).

In conclusion, the addition of exopolysaccharide ID1 to the vitrification media improves the cryotolerance of IVP bovine blastocysts to VitTrans vitrification by increasing embryo post-warming survival and hatchability. Furthermore, EPS ID1 addition may improve post-warming quality of blastocysts by preserving *BAX* and upregulating *GPX1* and *CDX2* gene expression, keeping the embryo´s potential for implantation after one-step warming and direct transfer.

AUTHOR CONTRIBUTIONS

I.M.-R. and T.M. conceived and designed the experiments; I.M.-R., A.S.-H. and A.O.-L. performed the experiments; C.O.-H., M.Y. E.M. and T.M. provided the resources, I.M.-R., A.S.-H. and M.Y. analysed the data; I.M.-R. and T.M. wrote the manuscript.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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