

# A shorter equilibration period in the VitTrans in-straw bovine embryo vitrification method improves post-warming outcomes

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## Research

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# Abstract

## Background

VitTrans is a device that enables the vitrification and warming/dilution of *in vitro* produced bovine embryos followed by their direct transfer to recipient females in field conditions. This study sought to improve the VitTrans method by comparing two equilibration times: short (SE: 3 min) and long (LE: 12 min). Outcome measures recorded in vitrified D7 and D8 expanded blastocysts were survival and hatching rates, differential cell counts, apoptosis rate and gene expression.

## Results

While survival rates at 3 h and 24 h post-warming were reduced ( $P < 0.05$ ) after vitrification, hatching rates of D7 embryos vitrified after SE were similar to those obtained in fresh non-vitrified blastocysts. Hatching rates of vitrified D8 blastocysts were lower ( $P < 0.05$ ) than of fresh controls, regardless of treatment. Total cell counts, and inner cell mass and trophectoderm cell numbers were similar in hatched blastocysts derived from D7 blastocysts vitrified after SE and fresh blastocysts, while vitrified D8 blastocysts yielded lower values, regardless of treatment. The rate of apoptotic cells was significantly higher in both treatment groups when compared to fresh controls, although apoptosis rates were lower using the SE than LE protocol. No differences emerged in expression of the genes BAX, AQP3, CX43 and IFN $\tau$  between blastocysts vitrified after SE or LE, whereas a significantly higher abundance of BCL2L1 and SOD1 transcripts was observed in blastocysts vitrified after SE compared to LE.

## Conclusions

The VitTrans device combined with a shorter exposure to the equilibration medium improves vitrification/warming outcomes facilitating the direct transfer of vitrified embryos under field conditions.

## Background

In beef and dairy cattle, *in vitro* embryo production (IVP) through assisted reproductive technologies is gaining popularity as an alternative to artificial insemination and *in vivo* embryo transfer to improve genetic gains. This approach also helps circumvent breeding problems such as cows that may not ovulate or show compromised fertility during periods of heat stress (reviewed by [1]). Because of the large numbers of embryos generated through *in vitro* technologies, the cryopreservation of these embryos has become an important topic of research. Studies have shown that *in vivo*-derived transferable-stage embryos of many mammalian species can be successfully preserved through conventional slow freezing. In contrast, vitrification seems the most effective method for embryos produced *in vitro*, as they are highly susceptible to cryoinjury [2]. While vitrification is simpler, faster and cheaper than slow cryopreservation methods, it requires higher concentrations of cryoprotectant agents (CPAs) which could have deleterious effects on embryo development after their warming. To minimize this effect, warming is achieved via a complex dilution procedure along with the use of a stereomicroscope to completely remove the vitrification solution. When working under farm conditions, this procedure is especially technically demanding.

When using vitrification technology in veterinary practice, a practical approach is needed for the warming of vitrified embryos so that embryos can be directly and easily transferred to the uterus. So far, there have been several attempts to replace successive dilution steps with one-step in-straw cryoprotectant dilution [3–14]. However, in some of these

procedures, in-straw embryo warming requires more than one dilution step and proper handling of the carrier system, demanding more accuracy when these techniques are to be used in the field by embryo-transfer practitioners [7, 9, 10, 12]. Using the device VitTrans designed by our group, IVP embryos are easily warmed/diluted in-straw for their transfer to recipient females in field conditions [15]. The performance of VitTrans assessed in terms of post-warming survival rates after 24 h of culture of IVP bovine embryos is comparable to that observed with our control vitrification-warming method [15].

For the vitrification of a solution, a radical increase in both the cooling rate and cryoprotectant concentration is required. Unfortunately, most cryoprotectants have some negative effects, including toxicity and osmotic injury. Although there is no consensus regarding the toxicity of penetrating CPAs, it is widely accepted that the higher their concentration and higher the exposure temperature, the greater their toxicity. Hence, any variation in exposure time prior to cooling can cause dramatic differences in cellular hydration [16, 17]. In any vitrification protocol it is accordingly important to achieve an adequate balance between obtaining a high level of dehydration and high viscosity while also avoiding toxicity. The first step is usually an equilibration stage in a solution containing a relatively low CPA concentration, followed by ultra-short (30–90 s) exposure to a vitrification medium with a higher concentration of cryoprotectant (usually double the initial concentration) and dehydrating agent (usually, a disaccharide, such as sucrose). Exposure to the equilibration medium may be short (e.g., 1 min followed by 25 s during vitrification) [12, 18] or last for 3 min followed by vitrification for 25 s [9, 19, 20] or even longer (e.g., 10–15 min followed by vitrification for 60 s) [21]. These durations have provided adequate blastocyst survival, and hatching and pregnancy rates.

Given this background, we hypothesized that a shorter exposure time only in the first step of the VitTrans protocol could be a safe approach to allow for delivery of the cryoprotectants to the blastocyst, thereby minimizing the likelihood of toxicity or osmotic damage. The objective of the present study was to determine whether a first equilibration step of the VitTrans protocol shortened from 12 min to 3 min would serve to improve the post-warming quality of vitrified day 7 and day 8 expanded blastocysts. Outcomes were assessed in terms of survival rates, differential cell counts, cell apoptosis and relative abundances of mRNAs of genes involved in apoptosis, oxidative-stress pathways, water channels, implantation and gap junctions recorded in the warmed embryos.

## Methods

### Chemicals and suppliers

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (Mo, USA).

### In vitro production of bovine blastocysts

Embryos were produced according to our previously established procedures [22], with minor modifications. Briefly, cow ovaries were collected at a local abattoir and transported to the laboratory in saline solution (0.9% NaCl) at 35–37 °C. Cumulus oocyte complexes (COCs) were obtained by aspiration from 3– to 8– mm follicles, and only COCs with three or more layers of cumulus cells and a homogeneous cytoplasm were selected for *in vitro* maturation (IVM). After three washes in modified Dulbecco's PBS (phosphate-buffered saline) (PBS supplemented with 36 mg mL<sup>-1</sup> pyruvate, 50 mg mL<sup>-1</sup> gentamicin and 0.5 mg mL<sup>-1</sup> bovine serum albumin, (BSA)), groups of 40 to 50 COCs were transferred to 500 µL of maturation medium in four-well plates and cultured for 24 h at 38.5 °C in a 5% CO<sub>2</sub> humidified air atmosphere. The maturation medium consisted of TCM-199 supplemented with 10% (v/v) foetal calf serum (FCS), 10 ng mL<sup>-1</sup> epidermal growth factor and 50 mg mL<sup>-1</sup> gentamicin.

For *in vitro* fertilization (IVF), motile spermatozoa were obtained by centrifuging frozen-thawed sperm from one Asturian bull (ASEAVA, Llanera, Asturias, Spain) of proven fertility at 300 x g for 10 min on a discontinuous gradient composed of 1 mL 40% Bovipure on 1 mL of 80% BoviPure (Nidacon Laboratories AB, Göthenborg, Sweden). The underlying sperm pellet was resuspended in 3 mL of BoviWash (Nidacon International, Gothenburg, Sweden) and pelleted by centrifugation at 300 x g for 5 min. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg mL<sup>-1</sup> fatty acid-free BSA and 10 mg mL<sup>-1</sup> heparin-sodium salt) to a final concentration of 2 × 10<sup>6</sup> spermatozoa mL<sup>-1</sup>. Groups of 40 to 50 *in vitro* matured oocytes were transferred to 250 µL of IVF medium and co-incubated with 250 µL of sperm suspension (final concentration of 1 × 10<sup>6</sup> spermatozoa mL<sup>-1</sup>) at 38.5 °C in a 5% CO<sub>2</sub> humidified air atmosphere.

At approximately 18 h post-insemination (hpi), presumptive zygotes were denuded by gentle pipetting and transferred to 25-µL culture drops (1 embryo/µL) under mineral oil. The culture medium was Synthetic Oviduct Fluid (Caisson Labs, Smithfield, USA) supplemented with 0.96 µg mL<sup>-1</sup> BSA, 88.6 µg mL<sup>-1</sup> Na-pyruvate, 2% (v/v) non-essential amino acids, 1% (v/v) essential amino acids, 0.5% (v/v) gentamicin and 2% (v/v) FCS. Plates were incubated for 7 or 8 days at 38 °C in a 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> humidified air atmosphere. Cleavage rates were recorded 48 h after insemination and the number of blastocysts was determined on Days 7 and 8 after insemination.

## Embryo vitrification and warming

Blastocysts were vitrified using the VitTrans device and vitrification and warming solutions as previously described by Morató and Mogas [15]. The VitTrans consists of a carrier where the embryo is loaded, a hard plastic handle and a covering straw, which protects the device from mechanical damage during storage and serves as a 0.5-mL straw for sample dilution during warming and embryo transfer. The handle has an inner channel through which warming solutions are introduced to dilute the cryoprotectant and displace the embryo to the straw for transfer (Fig. 1). The holding medium (HM) used to formulate the vitrification-warming solutions was TCM-199 HEPES with 20% (v/v) FCS. All steps were performed under a laminar flow hood heated to 38.5 °C using a stereomicroscope to visualize each step.

## Vitrification protocol

Day 7 (D7) and Day 8 (D8) blastocysts were randomly transferred to an equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO) in HM for 3 min (short equilibration: SE) or 12 min (long equilibration: LE). The blastocysts were then transferred to vitrification solution (VS) containing 15% (v/v) EG + 15% (v/v) DMSO + 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, embryos (up to 2) were loaded onto the embryo attachment piece of the VitTrans device, almost all the solution removed to leave only a thin layer covering the blastocysts and the sample quickly plunged in liquid nitrogen. Subsequently, the VitTrans device was covered with the 0.5-mL plastic straw. The entire process from immersion in VS to plunging in liquid nitrogen was completed within 1 min. The loaded devices were stored in liquid nitrogen.

## Warming protocol

For warming, the cover of the VitTrans inside liquid nitrogen was twisted open for 10 s to release pressure. Then, the whole VitTrans device (with its cover) was removed from the liquid nitrogen, held for 1 s in air and submerged in a water bath at 45 °C for 3 s, leaving the hard handle above the water surface. While in the water bath, a syringe containing the diluting solution (0.5 M sucrose in HM) at 45 °C was connected to the hard handle using the Luer connector. Next, the whole VitTrans device (with its cover) was removed from the water bath as the diluting solution

was injected through the lumen of the device. Once the diluting solution entered the straw, the outside was dried to remove any remaining water, and the VitTrans removed from the straw. At this point, the straw containing the warmed embryos is ready for transfer.

To determine embryo survival in subsequent experiments, the cotton plug end of the straw was cut and the contents of the straw expelled into a Petri dish. Blastocysts were then transferred to the culture medium and incubated at 38 °C in a 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> humidified air atmosphere for 24 h. Survival rates were expressed as proportions of blastocysts showing signs of re-expansion at 3 and 24 h post-warming. Hatching rates were defined as proportions of hatching/hatched blastocysts at 24 h post-warming (Experiment 1; 9 replicates). Fresh non-vitrified D7 or D8 blastocysts served as non-vitrified controls. The expanded and hatching/hatched blastocysts from groups D7-Control, D7-SE, D7-LE, D8-Control, D8-SE, and D8-LE were fixed and immunostained to determine the variables: total cell number (TCN), inner cell mass (ICM) cell number, trophectoderm (TE) cell number and apoptosis rate (AR) (Experiment 2; 4 replicates). Surviving expanded and hatching/hatched blastocysts from D7-Control, D7-SE, and D7-LE were pooled in groups of 5 blastocysts, snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction and RT-qPCR analysis (Experiment 3; 5 replicates).

## Differential staining and TUNEL

At 24 h post-warming, expanded and hatched blastocysts surviving vitrification in each group underwent immunostaining plus the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay to quantify TCN, ICM cell number, TE cell number and AR. Fresh non-vitrified D8 blastocysts served as controls. The protocol for embryo staining was based on Vendrell-Flotats *et al.* [23] with some modifications. All steps were done at 38.5 °C unless otherwise stated. Blastocysts were fixed in 2% (v/v) paraformaldehyde in PBS for 15 min at room temperature (RT). After fixation, embryos were washed at least three times in PBS and permeabilised in 0.01% Triton X-100 in PBS supplemented with 5% normal donkey serum (PBS-NDS) for 1 h at RT. The embryos were washed in PBS (3×) and incubated at 4 °C overnight with mouse anti-SOX2 primary antibody (1:100; Invitrogen, CA, USA) in a humidified chamber. Next, the embryos were washed in PBS (3×) for 20 min and permeabilised again with 0.005% Triton X-100 in PBS-NDS for 20 min. The embryos were then incubated with the secondary antibody goat anti-mouse IgG Alexa Fluor 568 (1:500; ThermoFisher, MA, USA) diluted for 1 h in a humidified chamber. They were then transferred to PBS-NDS-0.005% Triton X-100 for 20 min, washed in PBS (3×) and incubated in the TUNEL ( ) reaction mixture dilution following the manufacturer's instructions (in situ Cell Death Detection Kit, Fluorescein) for 1 h in the dark. Positive and negative control samples were included in each assay. Blastocysts exposed to DNase I for 15 min at RT served as positive controls and blastocysts not exposed to the terminal TdT enzyme served as negative controls. Embryos were then washed thoroughly in 0.005% Triton X-100 in PBS-NDS for 5 min, mounted on poly-L-lysine treated coverslips fitted with a self-adhesive reinforcement ring in a 3-μL drop of Vectashield containing 125 ng mL<sup>-1</sup> 4',6-diamidino-2-phenylindole (DAPI) (Vectorlabs, Burlingame, CA), and flattened with a slide. The preparation was sealed with nail varnish and stored at 4 °C protected from light until observation within the following 3 days. Confocal images in serial sections separated by 0.5 μm were captured with a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) to examine the ICM cell nuclei (SOX2-Alexa Fluor 568; excitation 561 nm), cell nucleus (DAPI; excitation 405 nm) and DNA fragmentation (fluorescein isothiocyanate-conjugated TUNEL label; excitation 488 nm). TCN, ICM cell number, and apoptotic cells were analysed using Imaris 9.2 software (Oxford Instruments, UK). Individual nuclei were counted as intact (TUNEL(-); blue/red stain) or fragmented (TUNEL(+), green stain) DNA, TE cells (SOX2(-); blue stain) or ICM cells (SOX2(+); red stain) (Fig. 2). The total number of cells was calculated as the sum of the TE and ICM cells. The AR was calculated as the ratio TUNEL(+) cells/total number of cells.

DAPI (406-diamidino-2-phenylindole), TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling).

## RNA extraction, reverse transcription and quantitative real-time PCR

Gene expression analysis was performed 24 h after warming following the procedures used for RNA extraction and real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) previously described by [24]. Blastocysts were washed three times in Dulbecco's PBS containing 0.3% polyvinyl alcohol (PVA) at 38.5 °C and then pipetted in pools of 5 embryos into 0.5 mL microtubes. Tubes were then immediately plunged in liquid nitrogen and stored at - 80 °C until further processing.

Poly-(A)-RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (DynaL Biotech, Oslo, Norway) following the manufacturer's instructions with minor modifications. For poly-(A)-RNA extraction, each pool of blastocysts was lysed in 50 µL of lysis buffer at RT for 5 min by gently pipetting, and the fluid lysate was then hybridized with 10 µL of prewashed beads for 5 min at RT by gently shaking. After hybridization, poly-(A)-RNA-bead complexes were washed at RT twice in 50 µL of Washing Buffer A and two more times in 50 µL of Washing Buffer B. Next, the samples were eluted in 16 µL of Elution Buffer (Tris HCl) and heated to 70 °C for 5 min. Immediately after extraction, 4 µL of qScript cDNAsupermix (Quanta Biosciences; Gaithersburg, MD, USA) was added and the Reverse Transcription (RT) reaction carried out using oligo-dT primers, random primers, dNTPs and qScript reverse transcriptase. The RT reaction was run for 5 min at 25 °C, followed by 1 h at 42 °C to allow the RT-qPCR of mRNA, and 10 min at 70 °C to denature the reverse transcriptase enzyme. After RT, the resulting cDNA was diluted in 25 µL of Tris HCl (elution solution).

The relative abundance of mRNA transcripts was quantified by the qPCR method using a 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The qPCR reaction mix contained 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA), 1.2 µL of each primer (500 nM; Life Techno Life Technologies, Madrid, Spain) and 2 µL of cDNA template. Nuclease-free water was added to a final volume of 20 µL. PCR amplification consisted of one cycle of denaturation at 95 °C for 10 min, 45 cycles of amplification with a denaturation step at 95 °C for 15 seconds, an annealing step for 1 min at 60 °C (the appropriate annealing temperature of the primers) and a final extension step at 72 °C for 40 s. Fluorescence data were acquired during the final extension step. The identity of the amplified PCR product was verified by melting curve analysis and gel electrophoresis (on a 2% agarose gel containing 0.1 µg/mL SafeView; Applied Biological Materials, Vancouver, Canada). The melting protocol consisted of heating the samples from 50 to 95 °C and holding at each temperature for 5 s while monitoring fluorescence. In each run there were three technical replicates from each of the four biological replicates per individual gene. Further, negative controls were also included for the template and reverse transcription and amplified by PCR to ensure no cross-contamination.

The comparative threshold cycle (Ct) method was used to quantify the relative expression of six candidate genes (*BAX*, *BCL2L1*, *AQP3*, *SOD1*, *CX43* and *IFN $\tau$* ) in vitrified/warmed viable blastocysts at 24 h post-warming, normalized to the endogenous control housekeeping (HK) genes *PPIA* and *H3F3A*. Fluorescence data were acquired after each elongation step to determine the threshold cycle for each sample. The threshold cycle, which is set in the log-linear phase, reflects the PCR cycle number at which the fluorescence generated within a given reaction is just above background fluorescence. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative Ct method, the  $\Delta$ Ct value was determined by subtracting the mean between *PPIA* and *H3F3A* Ct values for each sample from the Ct value of each target gene of the sample for each replicate separately. Calculation of  $\Delta\Delta$ Ct involved the subtraction of the  $\Delta$ Ct value for the average  $\Delta$ Ct across all embryos per target. Fold differences in relative transcript abundances were calculated for

target genes assuming an amplification efficiency of 100% and using the formula  $2^{-(\Delta\Delta Ct)}$ . Primer sequences, amplicon size and GenBank accession numbers for each gene are provided in Table 1. The efficiency of primer amplification was 100%. Non-template controls were not amplified or returned a Ct value 10 points higher than the average Ct value for the genes. The experiment was repeated independently four times.

Table 1  
Primers used for reverse transcription-quantitative polymerase chain reaction.

Symbol	Primer sequences (5'-3')	Amplicon size (bp)	GenBank Accession no.
BCL2 associated X apoptosis regulator ( <i>BAX</i> )	F: ACCAAGAAGCTGAGCGAGTG R: CGGAAAAAGACCTCTCGGGG	116	NM_173894.1
BCL2-like 1 ( <i>BCL2L1</i> )	F: GAGTTCGGAGGGGTCATGTG R: TGAGCAGTGCCTTCAGAGAC	211	NM_001166486.1
Superoxide dismutase 1 ( <i>SOD1</i> )	F: ACACAAGGCTGTACCAGTGC R: CACATTGCCAGGTCTCCA	102	NM_174615.2
Aquaporin 3 ( <i>AQP3</i> )	F: GTGGACCCCTACAACAACCC R: CAGGAGCGGAGAGACAATGG	222	NM_001079794.1
Connexin 43 ( <i>CX43</i> )	F: TGGAATGCAAGAGAGGTTGAAAGAGG R: AACACTCTCCAGAACACATGATCG	294	NM_174068.2
Interferon tau ( <i>IFN<math>\tau</math></i> )	F: CTGAAGGTTACCCAGACCC R: GAGTCTGTTTCATTCGGGCCA	197	AF238612
Peptidylprolyl isomerase A ( <i>PPIA</i> )	F: CACACAGGTCCTGGCATCTTGTC R: CACGTGCTTGCCATCCAACC	108	NM_178320.2
H3.3 histone A ( <i>H3F3A</i> )	F: CATGGCTCGTACAAAGCAGA R: ACCAGGCCTGTAACGATGAG	136	NM_001014389.2

## Statistical analysis

All statistical tests were performed using the statistical package IBM SPSS Version 21.0 for Windows (IBM Corp.; Chicago, Illinois, USA). The data were first checked for normality using the Shapiro-Wilk test, and for homogeneity of variances using the Levene test.

Survival rates were compared by two-way analysis of variance (ANOVA) followed by Sidak's test for pair-wise comparisons. Total cell counts, number of ICM cells, and apoptosis rate were analyzed by the three-factor general linear model. Relative transcript abundances were assessed by two-factor ANOVA followed by the post-hoc Sidak's test. When data were not normally distributed or variances were not homogenous, data were linearly transformed into arcsine square roots, square roots or logarithms. When transformed data did not fulfil parametric assumptions, Kruskal-Wallis and Mann-Whitney tests were used as non-parametric alternatives. Data are expressed as means  $\pm$  standard error of the mean (SEM). Significance was set at  $P \leq 0.05$ .

## Results

*A shorter time of exposure of embryos to the equilibrium solution leads to improved embryo development (Experiment 1)*

Post-warming survival and hatching rates of D7 and D8 expanded blastocysts vitrified after a short (3 min) or long (12 min) period of exposure to the equilibration solution are shown in Table 2. Vitrification led to significant reductions in D7 and D8 embryo survival rates recorded at 3 h or 24 h post-warming when compared to fresh control blastocysts. While no effects of the equilibration time were observed on embryo survival assessed at 3 h post-warming, both D7 and D8 vitrified blastocysts subjected to SE showed significantly higher survival and hatching rates ( $P < 0.05$ ) than those blastocysts vitrified after a longer equilibration period. Hatching rates of D7 blastocysts in the SE group did not differ from those observed for the fresh non-vitrified blastocysts ( $31.4 \pm 3.7$  vs.  $35.9 \pm 4.0$ , respectively). However, vitrified D8 blastocysts showed significantly lower hatching rates than those derived from fresh non-vitrified embryos, regardless of SE or LE.

In addition, survival at 24 h post-warming was significantly higher for the vitrified D7 blastocysts than D8 blastocysts, regardless of the equilibration period. The SE treatment significantly increased the hatching capacity of vitrified D7 blastocysts when compared to vitrified D8 blastocysts. However, no differences in hatching rates were observed between D7 and D8 blastocysts vitrified after the LE treatment.

Table 2  
Post-warming survival and hatching rates of D7 and D8 expanded blastocysts vitrified after shorter or longer exposure to the equilibration solution

	Day 7 blastocysts				Day 8 blastocysts			
	n	Survival (%) (3 h)	Survival (%) (24 h)	Hatching rate (%) (24 h)	n	Survival (%) (3 h)	Survival (%) (24 h)	Hatching rate (%) (24 h)
Control	86	100 <sup>a,d</sup>	100 <sup>a,d</sup>	$35.9 \pm 4.0$ <sup>a,d</sup>	40	100 <sup>a,d</sup>	100 <sup>a,d</sup>	$50.0 \pm 7.0$ <sup>a,e</sup>
SE	86	$60.6 \pm 1.5$ <sup>b,d</sup>	$78.4 \pm 2.0$ <sup>b,d</sup>	$31.4 \pm 3.7$ <sup>a,d</sup>	33	$48.6 \pm 5.3$ <sup>b,e</sup>	$63.0 \pm 5.5$ <sup>b,e</sup>	$19.9 \pm 2.7$ <sup>b,e</sup>
LE	83	$57.5 \pm 4.0$ <sup>b,d</sup>	$63.1 \pm 2.6$ <sup>c,d</sup>	$10.1 \pm 2.4$ <sup>b,d</sup>	36	$39.4 \pm 4.7$ <sup>b,e</sup>	$55.3 \pm 5.0$ <sup>c,e</sup>	$8.1 \pm 2.7$ <sup>c,d</sup>

<sup>a,b,c</sup> Values within columns with different superscripts differ significantly ( $P < 0.05$ ); <sup>d,e</sup> Same values within rows with different superscripts differ significantly ( $P < 0.05$ ).

Data shown as mean  $\pm$  SEM.

Control: fresh non-vitrified expanded blastocysts; SE: expanded blastocysts vitrified after a short equilibration time (3 min); LE: expanded blastocysts vitrified after a long equilibration time (12 min).

*Different exposure times to the equilibration solution modify TCN, ICM cell numbers, TE cell numbers and apoptosis rates at 24 h post-warming (Experiment 2)*

The outcomes TCN, ICM and TE cell numbers and AR determined 24 h post-warming of D7 and D8 expanded bovine blastocysts vitrified after the short and long equilibration times are shown in Table 3. TCN and TE cell numbers were

significantly lower in expanded blastocysts derived from vitrified/warmed D7 blastocysts compared to those derived from fresh control blastocysts, regardless of the length of exposure to the equilibration solution. However, both outcome measures were similar in non-vitrified fresh and SE-vitrified D7 blastocysts reaching the hatching stage at 24 h post-warming, while they were significantly lower in LE-vitrified D7 blastocysts. The rate of apoptotic cells was significantly higher in both vitrification groups when compared to fresh controls, although vitrification using the SE protocol produced less apoptosis than when the LE protocol was used.

No differences were observed when TCN and TE cell number were assessed at 24 h post-warming in expanded blastocysts derived from fresh D8 blastocysts or D8 blastocysts vitrified using the SE protocol. However, both counts were significantly lower in expanded blastocysts derived from D8 blastocysts vitrified using the LE protocol. ICM cell numbers in expanded blastocysts derived from vitrified/warmed D8 blastocysts were significantly lower compared to control fresh blastocysts, regardless of the vitrification protocol. A similar trend was observed for TCN, and ICM and TE cell numbers assessed in hatched blastocysts derived from vitrified/warmed D8 blastocysts. Apoptosis rates were significantly higher for vitrified/warmed D8 blastocysts when compared to non-vitrified embryos, although the SE protocol yielded a significantly lower apoptosis rate than the LE protocol.

Table 3

Total cell number, ICM and TE cell numbers and rate of apoptotic cells in warmed Day 7 and Day 8 expanded bovine blastocysts vitrified after a short or long exposure time to the equilibration solution

Day 7 blastocysts									
		TCN		ICM cell number		TE cell number		AR	
	n	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched
Control	30	140.3 ± 8.6 <sup>a,d</sup>	189.8 ± 4.4 <sup>a,e</sup>	24.4 ± 1.6 <sup>a,d</sup>	38.6 ± 1.6 <sup>a,e</sup>	115.9 ± 7.9 <sup>a,d</sup>	151.3 ± 4.1 <sup>a,e</sup>	3.7 ± 0.4 <sup>a</sup>	4.7 ± 0.7 <sup>a</sup>
SE	23	110.0 ± 2.7 <sup>b,d</sup>	195.2 ± 3.5 <sup>a,e</sup>	22.0 ± 2.1 <sup>a,d</sup>	35.1 ± 1.5 <sup>a,e</sup>	87.1 ± 2.3 <sup>b,d</sup>	160.1 ± 3.1 <sup>a,e</sup>	11.5 ± 1.1 <sup>b</sup>	9.1 ± 0.9 <sup>b</sup>
LE	21	113.2 ± 4.5 <sup>b,d</sup>	170.6 ± 1.4 <sup>b,e</sup>	23.3 ± 1.3 <sup>a,d</sup>	33.2 ± 3.3 <sup>a,e</sup>	89.9 ± 4.3 <sup>b,d</sup>	135.4 ± 2.8 <sup>b,e</sup>	15.2 ± 0.3 <sup>c</sup>	13.6 ± 1.2 <sup>c</sup>
Day 8 blastocysts									
		TCN		ICM cell number		TE cell number		AR	
	n	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched
Control	40	125.3 ± 5.4 <sup>a,d</sup>	206.8 ± 12 <sup>a,e</sup>	29.2 ± 1.9 <sup>a,d</sup>	43.1 ± 1.2 <sup>a,e</sup>	96.0 ± 4.5 <sup>a,d</sup>	163.6 ± 5.6 <sup>a,e</sup>	5.6 ± 0.4 <sup>a</sup>	4.6 ± 0.7 <sup>a</sup>
SE	21	128.1 ± 2.8 <sup>a,d</sup>	169.7 ± 5.6 <sup>b,e</sup>	22.2 ± 1.0 <sup>b,d</sup>	32.0 ± 1.2 <sup>b,e</sup>	105.9 ± 2.8 <sup>a,d</sup>	137.8 ± 4.0 <sup>b,e</sup>	15.1 ± 0.6 <sup>b</sup>	12.7 ± 0.8 <sup>b</sup>
LE	20	108.4 ± 1.3 <sup>b,d</sup>	154.6 ± 2.1 <sup>b,e</sup>	19.7 ± 0.8 <sup>b,d</sup>	29.0 ± 2.0 <sup>b,e</sup>	88.7 ± 1.3 <sup>b,d</sup>	125.0 ± 2.7 <sup>b,e</sup>	25.1 ± 1.5 <sup>c</sup>	23.2 ± 2.1 <sup>c</sup>
<sup>a,b,c</sup> Values within columns with different superscripts differ significantly ( $P < 0.05$ ); <sup>d,e</sup> Values within rows with different superscripts differ significantly ( $P < 0.05$ ).									
Data shown as mean ± SEM. TCN: total cell number; ICM: inner cell mass; TE: trophectoderm; AR: apoptosis rate. Expanded: expanded blastocyst; Hatched: hatching/hatched blastocyst.									

Control: fresh non-vitrified expanded blastocysts; SE: expanded blastocysts vitrified after a short equilibration period (3 min); LE: expanded blastocysts vitrified after a long equilibration period (12 min).

*Different times of exposure to the equilibration solution modify gene expression patterns in warmed expanded blastocysts vitrified using the VitTrans as the cryodevice (Experiment 3)*

Given the effects on embryo development and embryo quality observed in our initial experiments, the effects of the shorter and longer equilibration times on the relative abundance of genes was only assessed in post-warmed expanded and hatched blastocysts derived from vitrified/warmed D7 expanded embryos (Fig. 3). While no significant differences in *BAX* expression was observed between the two treatments, the *BCL2L1* gene was overexpressed in both expanded and hatched blastocysts derived from SE-vitrified blastocysts compared to blastocysts derived from fresh or LE-vitrified blastocysts. mRNA transcript abundances of the *SOD1* gene were significantly higher in blastocysts derived from SE- than LE-vitrification although *SOD1* mRNA abundances in both vitrification groups did not differ from those detected in blastocysts derived from fresh non-vitrified blastocysts. No differences in *AQP3*, *CX43* and *IFN $\tau$*  transcript abundances were observed between treatments. However, expanded and hatched blastocysts derived from blastocyst vitrified using the SE protocol showed a clear trend ( $P=0.07$ ) towards higher *CX43* and *AQP3* gene expression levels compared to expanded and hatched blastocysts vitrified using the LE protocol. When gene expression was compared between blastocyst stages, hatched blastocysts derived from vitrified blastocysts had higher *CX43* expression and lower *IFN $\tau$*  expression than their expanded counterparts, while no differences between the two stages were observed for the other genes.

Control: fresh non-vitrified expanded blastocysts; SE: D7 expanded blastocysts vitrified after a short equilibration time (3 min); LE: D7 expanded blastocysts vitrified after a long equilibration time (12 min).

## Discussion

To effectively transfer vitrification technology to the field, the procedures used for the warming and transfer of cryopreserved bovine embryos should be kept as simple as possible. The VitTrans device was designed to facilitate the vitrification/warming technique by including an easy one-step in-straw dilution method followed by direct embryo transfer to the uterus [15]. While we have reported post-warming survival rates of around 60% for D7 expanded blastocysts vitrified using VitTrans, here we modified the two-step vitrification protocol to improve post-warming outcomes. The objective of this study was to investigate the effects of different equilibration times on several post-warming outcome measures in bovine D7 and D8 expanded blastocysts vitrified using the VitTrans procedure. Our results indicate that a short equilibration time (3 min) during vitrification improves post-warming survival and the hatching ability of both D7 and D8 expanded blastocysts, whereas lengthening the equilibration time to 12 min does not seem to offer any further benefits. In addition, the hatching rates of D7-blastocysts vitrified by the SE protocol were similar to those recorded for fresh non-vitrified embryos. Several studies have compared equilibration times used in the vitrification of *in vitro* produced blastocysts of different species [25–29]. In cattle, Do *et al.* [27] found similar re-expansion (24 h post-warming) and hatching rates (48 h post-warming) when bovine expanded blastocysts were vitrified after a short (3 min) or long equilibration (8 min) time possibly explained by differences in temperature and equilibration times. Thus, while the short equilibration time tested by Do *et al.* [27] was similar to ours (3 min at 37 °C), their long equilibration protocol consisted of 8 min at room temperature, which may have resulted in reduced cytotoxicity and osmotic stress [16] and thus minimized differences between the use of their short or long protocol. Consistently, in a study carried out in the dromedary camel, loading of CPAs at 37 °C for a short exposure time (3 min) led to an outcome comparable to that of original processing at room temperature with a longer exposure time (15 min) [26]. When working at room temperature in humans and mice, different equilibration times did not affect

post-warming embryo survival [25, 28]. However, lengthening the exposure time to the equilibration solution from 4 to 8 min was found to improve the DNA integrity index after the vitrification of murine blastocysts [25, 28]. Prior to the vitrification of human blastocysts, 9–10 min of exposure to the equilibration solution improved the outcomes clinical pregnancy, embryo implantation and live birth rates compared to shorter exposure times [25].

Different vitrification outcomes have been recently reported after vitrification of expanded blastocysts using various one-step warming devices and short equilibration times. As we have also observed, one-step in-straw warming/dilution of expanded blastocysts vitrified in fiberplugs returned similar [9] or higher survival rates [20] for D7 than D8 blastocysts. However, either lower [9] or higher [20] hatching rates were observed at 24 h post-warming when D7 or D8 expanded blastocysts were vitrified in fiberplugs compared to our results. Further, one-step warming of bovine D7 expanded blastocysts vitrified on hand-pulled glass micropipettes as the cryodevice led to higher hatching rates assessed at 72 h post-warming rather than 24 h post-warming [12, 19].

When 24 h post-warming outcomes were compared after the vitrification of blastocysts produced after different times of *in vitro* culture, our results are consistent with those of others. Thus, significantly higher survival, re-expansion and hatching rates have been described after the vitrification of D7 compared to D8 IVP bovine blastocysts [20, 21, 30, 31] such that cryotolerance diminishes as the length of embryo culture increases. In the present study, although the hatching ability of D7 blastocysts vitrified/warmed within the SE protocol was comparable that of fresh non-vitrified D7 blastocysts, Day 8 vitrified/warmed blastocyst gave rise to under half of the hatching yield observed in the fresh control group. Early developing embryos are better at surviving than later embryos. This has been highlighted in prior work in which vitrified/warmed earlier cryopreserved IVP bovine blastocysts returned higher survival, hatching and pregnancy rates [20, 21].

The correct distribution of cells in the ICM and outer TE layer of the blastocyst is crucial for embryo development. However, while it is accepted that a minimal number of embryonic cells is needed to establish pregnancy [32], optimal ICM and TE cell numbers and distributions in the blastocyst remain unclear. Thus, higher ICM cell counts may lead to increased pregnancy rates [33] and an excessive number of cells allocated to the TE may lead to pregnancy abnormalities [34, 35]. Here, the TUNEL assay combined with differential staining for ICM and TE cells revealed significantly lower TCN and TE-cell numbers and a higher apoptosis rate in vitrified/warmed D7 re-expanded blastocysts compared to fresh ones while no differences emerged in ICM cell numbers, suggesting that the main site of cryopreservation-related membrane damage was the trophectoderm. Similar [36] or reduced total cell counts have been reported after bovine embryo vitrification [37, 38], mainly due to a low cell count in the TE. This effect is consistent with a greater accumulation of lipids in the TE than ICM [39], as cytoplasmic lipid contents appear strongly related to survival of cryopreservation [2]. In contrast, Gomez *et al.* [40] described that vitrification seemed to exert a detrimental effect on the ICM, while TE cells survived cryopreservation in numbers comparable to those counted in embryos before vitrification. However, we detected no differences in TCN, or in ICM and TE cell numbers between D7 blastocysts vitrified after SE and fresh blastocysts, while D7 blastocysts vitrified after LE showed significantly lower TCN and numbers of TE cells. This suggests that D7 expanded blastocysts vitrified using our SE protocol suffered less cryodamage or were able to recover from any damage at 24 h post-warming, showing similar hatching rates and embryo quality as those of fresh ones. However, among the D8 embryos subjected to vitrification/warming, TCN, and ICM and TE cell numbers were significantly lower in hatching blastocysts when compared to fresh blastocysts, regardless of the equilibration time. The timing of blastocyst formation is a good marker of embryo quality determining that early-cavitating embryos are of better quality than later cavitating embryos in terms of total cell numbers, inner cell mass and trophectoderm cell distributions, and cryosurvival [30, 34]. While we still lack reliable blastocyst stage morphological predictors of competence after embryo transfer, it is accepted by many research

groups and commercial companies that best pregnancy rates are achieved after the transfer of day 7 expanded bovine blastocysts whether fresh or cryopreserved (reviewed by [41, 42]).

Apoptosis has been frequently used as a marker for embryo quality as high rates of apoptotic cells have been linked to the reduced developmental competence of both *in vivo* or *in vitro* produced embryos [43–45]. Vitrification requires adequate dehydration and a high viscosity across all blastomeres and blastocele which is difficult given the characteristics of the blastocyst (multicellularity, presence of blastocele with high water content). This determines that vitrification leads to a post-warming increase in apoptosis [46]. Our results revealed that both equilibration solution exposure times induced apoptosis in surviving blastocysts by the time of their re-expansion and hatching. However, while the apoptosis rate for D7 expanded blastocysts vitrified via the VitTrans LE protocol was similar to that reported previously by Morató and Mogas [15], the apoptotic cell rate was significantly higher for the LE than SE protocol or control embryos. This finding suggests that the high toxicity effect of CPAs produced at high temperature can be avoided to some extent by reducing the time of exposure to the cryoprotectant [16]. Moreover, D8 embryos induced higher percentages of apoptotic cells than D7 embryos, in agreement with results observed when expanded blastocysts were vitrified/warmed using a one-step direct transfer procedure [20].

When genes related to apoptosis were analysed, a significantly higher abundance of *BCL2L1* transcripts was observed in both expanded and hatched blastocysts derived from the SE protocol when compared to fresh embryos or those vitrified after the long equilibration period, while there were no differences in *BAX* gene expression among treatments. Yang and Rajamahendran [47] related a higher expression level of Bcl-2 to better quality embryos less prone to apoptosis. However, the levels of *BCL2L1* gene expression observed in our study were inconsistent with apoptosis levels assessed by TUNEL in fresh or vitrified D7 blastocysts, suggesting that apoptosis detected by TUNEL is independent of the expression of *BCL2L1* or *BAX* genes, as observed previously [48]. Similarly, mRNA levels of *SOD1* were upregulated after SE treatment, indicating that a shorter exposure time may reduce oxidative stress by improving the activity of antioxidant enzymes and improving the quality of vitrified/warmed embryos [49]. In addition, a trend although not significant ( $P=0.07$ ) was observed towards greater *CX43* and *AQP3* gene expression in blastocysts subjected to SE compared to LE. In effect, high expression of *CX43*, a gene related to cell compaction and adhesion [50], has been linked to better quality and more cryotolerant embryos [51]. *AQP3* plays an important role in the transport of cryoprotectants and fluids during the cryopreservation of bovine embryos [52]. The presence of mRNA encoding this protein has been also related to better embryo cryotolerance [53]. While not always significant, the differences in gene expression observed in surviving blastocysts derived from D7 blastocysts vitrified after the SE treatment could be indicative of better embryo quality. In effect, these blastocysts showed an improved hatching ability together with higher TCN, and TE cell numbers and a lower apoptosis rate.

## Conclusions

In conclusion, vitrification of IVP D7 bovine embryos using the VitTrans device with a brief 3 min exposure to the equilibration solution gave rise to post-warming outcomes comparable to those of fresh non-vitrified blastocysts. In addition, our gene expression analysis indicated that the SE treatment could lead to the production of more high-quality blastocysts, promoting the efficiency of embryo transfer. This strategy of shortening the exposure time to the equilibration medium within the VitTrans procedure could have important implications for commercial *in vitro* embryo transfer programs simplifying the use of this technique in field conditions. Future experiments are underway to establish the full survival potential of these cryopreserved embryos after their transfer to recipient cows.

## Abbreviations

**ANOVA**

analysis of variance

**AR**

apoptosis rate

**BSA**

bovine serum albumin

**cDNA**

copy DNA

**COCs**

Cumulus oocyte complexes

**CPAs**

cryoprotectant agents

**D7**

day 7

**D8**

day 8

**DAPI**

4',6-diamidino-2-phenylindole

**DMSO**

dimethylsulfoxide

**EG**

ethylene glycol

**ES**

equilibration solution

**FCS**

foetal calf serum

**HM**

holding medium

**hpi**

hours post-insemination

**ICM**

inner cell mass

**IVF**

*in vitro* fertilization

**IVM**

*in vitro* maturation

**IVP**

*in vitro* produced

**LE**

long equilibration

**mRNA**

messenger RNA

**mRNA**

messenger RNA

**NDS**

normal donkey serum

**PBS**

phosphate-buffered saline

**PVA**

polyvinyl alcohol

**RT**

room temperature

**RT-qPCR**

quantitative reverse transcription PCR

**SE**

short equilibration

**SEM**

standard error of the mean

**TCN**

total cell number

**TE**

trophectoderm

**TUNEL**

Terminal deoxynucleotidyl transferase dUTP nick end labeling

**VS**

vitrification solution

## **Declarations**

### ***Ethics approval and consent to participate***

Not applicable.

### ***Consent for publication***

Not applicable.

### ***Availability of data and material***

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### ***Competing interests***

The authors declare that they have no competing interests.

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### ***Authors' contributions***

Conceptualization, I.M.R, M.Y and T.M; Formal analysis, I.M.R and M.Y; Funding acquisition, M.L.B and T.M; Investigation, I.M.R , T.G.M, EA.O.L, M.V.F; Methodology, I.M.R , T.G.M, EA.O.L, C. O. H and X.M; Project administration, M.L.B and T.M; Resources, J.E, S.A, M.L.B and T.M; Supervision, M.Y and T.M; Validation, M.Y and T.M; Writing – original draft, I.M.R; Writing – review & editing, T.M.

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Not applicable.

### ***Authors' information (optional)***

Not applicable.

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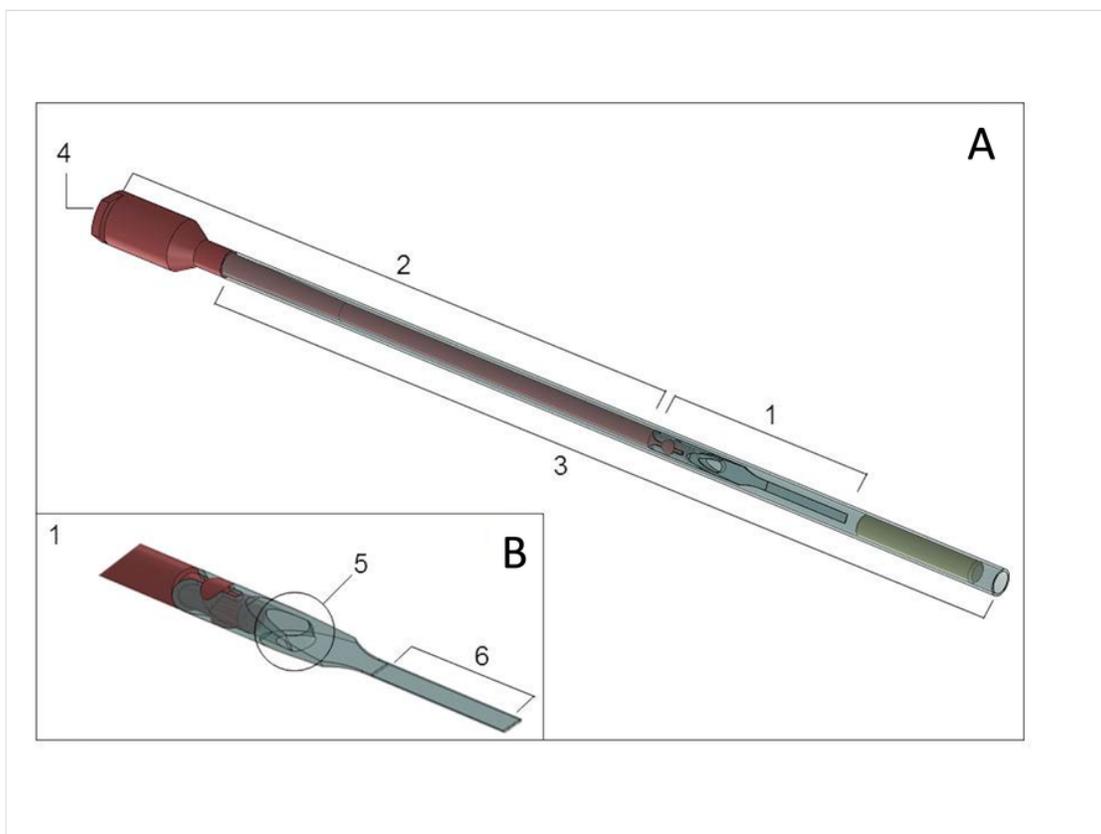
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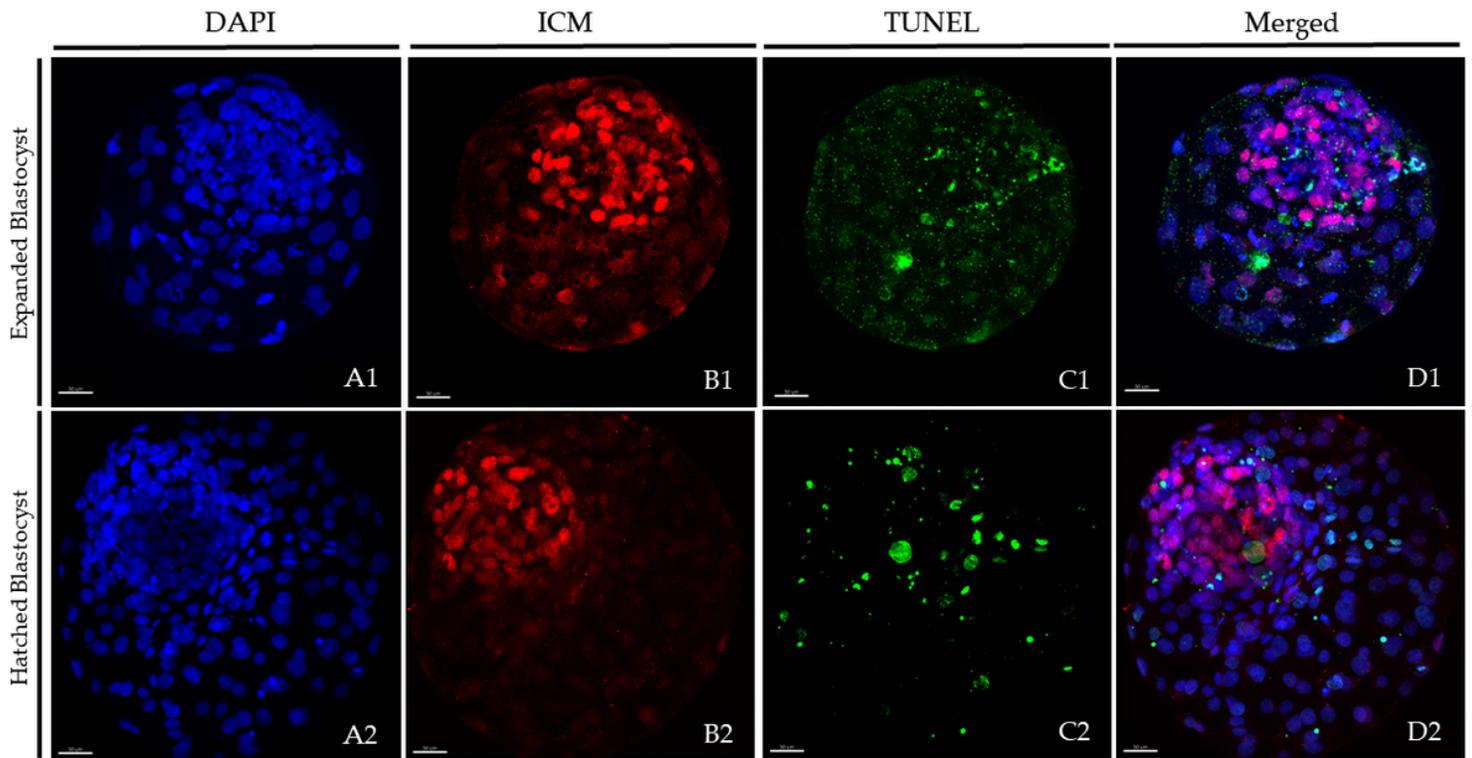
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## Figures



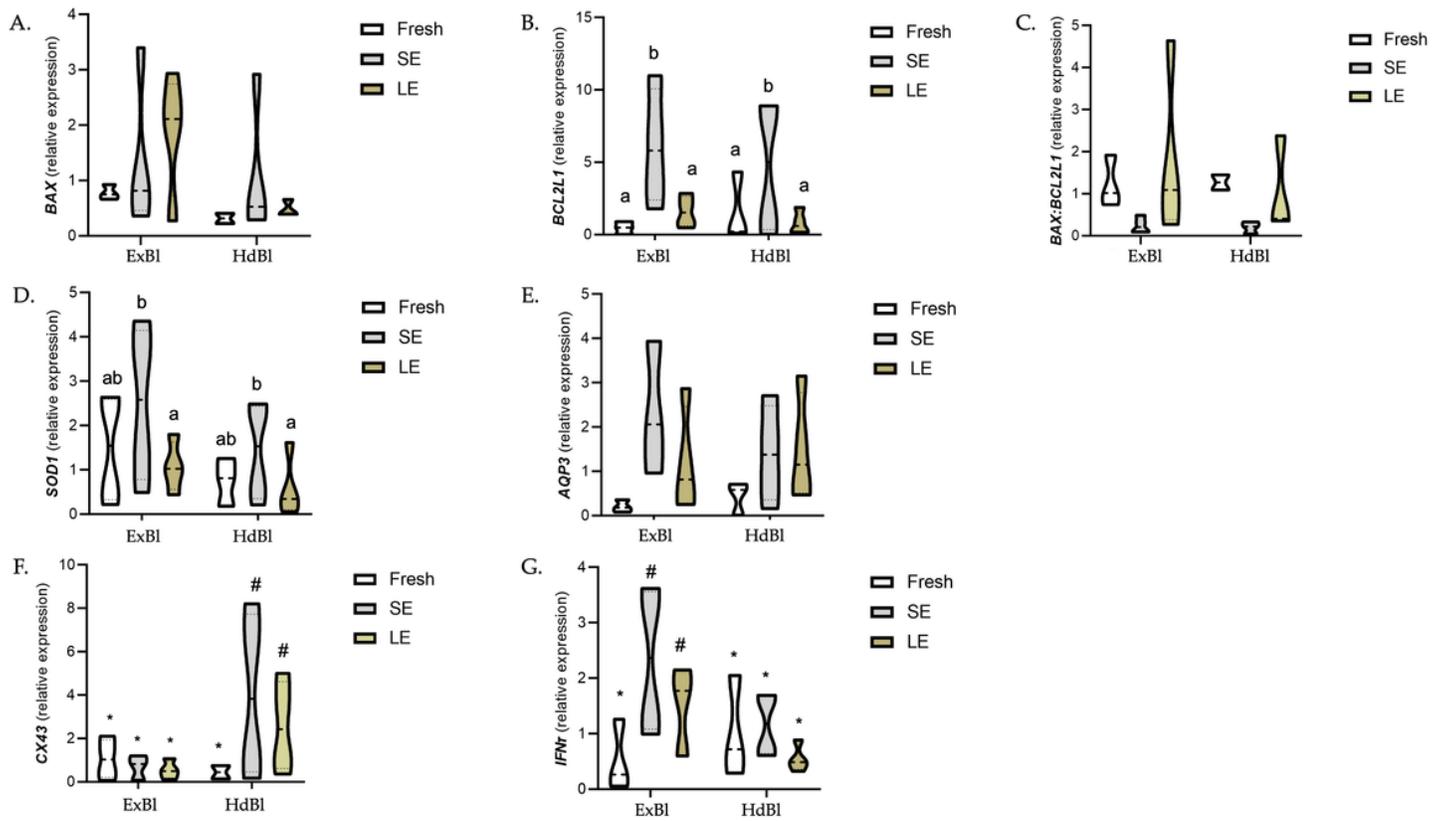
**Figure 1**

(A) The VitTrans comprises a carrier where the embryo is loaded (1), a hard plastic handle with an inner channel (2) into which warming solutions are introduced to dilute the cryoprotectant and transport the embryo to the straw (3) for transfer, and a Luer syringe connector (4) to connect the device to the warming solution source. The straw (3) 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. Scale bar: 2 cm. (B): Closer view of the end of the device (1) showing the outflow of the inner channel (5) and embryo attachment piece (6). Scale bar: 1 cm.



**Figure 2**

Representative pictures of post-warmed expanded and hatched blastocysts vitrified after short or long equilibration in the VitTrans procedure. After 24 h of culture post-warming, blastocysts were subjected to the TUNEL technique combined with differential staining. DAPI (blue), SOX2 (red) and TUNEL (green) staining were examined using DAPI, SOX2-Alexa Fluor and FITC filters respectively, for total (A1, A2), ICM (B1, B2) and apoptotic (C1, C2) cell counts. An overlay is provided in D1 and D2. A1, B1, C1 and D1: Expanded blastocysts; A2, B2, C2 and D2: Hatched blastocysts. Scale bar: 30  $\mu$ m. DAPI (406-diamidino-2-phenylindole), TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling).



**Figure 3**

Violin plots (solid line indicates the 50% quantile) showing expression levels of selected genes in post-warmed, expanded and hatched blastocysts derived from Day 7 expanded bovine blastocysts vitrified after a short or long time of exposure to the equilibration solution. Different letters indicate significant differences between treatments ( $P < 0.05$ ), and different symbols indicate differences between developmental stages for each specific treatment. BAX, BCL2 associated X apoptosis regulator; BCL2L1, BCL2 like 1; SOD1, superoxide dismutase 1; AQP3, aquaporin 3; CX43, connexin 43; IFN $\tau$ , interferon tau. ExBl, expanded blastocysts; HdBl, hatching/hatched blastocysts. Control: fresh non-vitrified expanded blastocysts; SE: D7 expanded blastocysts vitrified after a short equilibration time (3 min); LE: D7 expanded blastocysts vitrified after a long equilibration time (12 min).