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Original Research Article

Blastocoel fluid aspiration improves vitrification outcomes and produces similar sexing results of *in vitro*-produced cattle embryos compared to microblade biopsy

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ABSTRACT

The potential applications of *in vitro*-produced (IVP) cattle embryos are significantly enhanced when combined with genotype selection and cryopreservation techniques. While trophectoderm (TE) biopsies are frequently used for genotyping, cell-free DNA (cfDNA) found in blastocoele fluid (BF) arises as a less-invasive method. Moreover, the blastocoel collapse produced by BF aspiration could be beneficial for embryo cryotolerance. This study was conducted to test the BF as a source of cell free-DNA (cfDNA) and to compare the BF to the TE biopsy in terms of sexing efficiency/accuracy, embryo survival and gene expression after vitrification/warming. IVP day 7 expanded blastocysts were artificially collapsed by aspiration of BF (VIT-Collapsed) or biopsied (VIT-Biopsied). After sample collection, embryos were vitrified/warmed by the Cryotop method and individually cultured *in vitro*. Intact fresh non-vitrified and vitrified/warmed blastocysts served as Fresh Control and VIT-Control, respectively. After sex identification of BF or TE biopsies and the corresponding surviving embryos, amplification efficiency and sexing accuracy were assessed. There were no differences between the BF and TE biopsy samples in terms of sexing accuracy or efficiency. Although all vitrified groups showed lower post-warming re-expansion rates ($p < 0.05$), the blastocyst re-expansion rates in the VIT-Collapsed group were comparable to those in the Fresh Control group whereas biopsied blastocysts showed the lowest ($p < 0.05$) re-expansion rates. VIT-Collapsed blastocysts had hatching rates that were comparable to those of Fresh Control blastocysts but significantly higher than those of the other vitrification treatments. Proapoptotic gene *BAX* was overexpressed in VIT-Biopsied embryos, whereas *BCL2* transcripts were more abundant in the VIT-Collapsed group. On the other hand, VIT-Biopsied embryos showed altered *ATP1B1*- and *AQP3*-mRNA levels. The analysis of the cfDNA present in the BF is an efficient, minimally invasive approach to sex IVP cattle embryos. Besides, the artificial collapse of blastocoel prior to vitrification resulted in higher re-expansion and hatching ability than when embryos were vitrified after being biopsied.

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1. Introduction

In the cattle industry, *in vitro* embryo production (IVP) through assisted reproductive technologies has grown in popularity as a complement to artificial insemination and *in vivo* embryo transfer, offering a means to improve genetic gains or avoid periods of compromised fertility [1,2]. The use of IVP is increasing every year, as confirmed by the annual reports of the International Embryo Technology Society [3] and the Association of Embryo Technology in Europe [4]. When IVP is combined with genomic selection, animals can be chosen for breeding based on their genomic breeding values, such as lactation or carcass characteristics. The optimal use of genomic selection in an intensive embryo breeding program will accelerate the rate of genetic gain by further increasing selection intensity and drastically reducing the generation interval [5–7]. Besides calculating a genomic breeding value, the sex of the embryo can be determined. This sex determination can assist decisions in breeding programs to specifically transfer embryos of the desired sex because the economic worth of livestock depends on whether they are females (*i.e.,* for dairy cattle or breeding) or males (*i.e.,* for beef operations) [6]. Furthermore, an effective and reliable method to cryopreserve genotyped embryos is necessary for the preimplantation of a genomic selection system. In addition, cryopreservation systems contribute to the best use of surplus IVP embryos and to the lower-cost exchange of superior livestock compared to entire animal transportation while ensuring biosafety, animal welfare, and quality [8].

Genotype selection is based on the nuclear DNA present in cells obtained through the biopsy of a preimplantation embryo. There are, however, certain technical limitations as to how genomic screening or cryopreservation of preimplantation embryos could be practical enough to be widely used [5]. To maximize the possibilities that IVP of embryos offers, robust and practical methods for embryo biopsy and cryopreservation must be developed. Regarding the biopsy procedure, the bottleneck is how to manipulate preimplantation embryos to obtain a few cells with an intact nucleus, being gently enough to not decrease embryo viability or pregnancy rates [9,10] and collecting sufficient good-quality DNA for further genetic analysis. In cattle, pregnancy rates produced by biopsied and frozen-thawed IVP embryos are still suboptimal, and therefore, cryopreservation of biopsied embryos is mainly used for embryos produced *in vivo*, which are less sensitive to cryodamage than those *in vitro*-derived [11,12]. As vitrification overcomes the cell damage occurring in slow freezing due to ice crystals formation, it has become a more popular option to cryopreserve IVP embryos in the last decades. In fact, vitrification performs better than slow freezing in the case of biopsied embryos [13].

It is well established that the fully expanded blastocyst stage at day 7 (D7) is preferred for embryo cryopreservation, as post-warming outcomes are higher compared to other developmental stages [14,15]. Yet, the high volume of BF present inside the blastocoel cavity may make it more challenging for expanded blastocysts to survive vitrification due to osmotic changes during blastocysts' exposure to CPAs and formation of ice crystals during vitrification [16]. Previous studies in cattle [17,18] and other species, such as humans [16,19,20], horses [21], mice [22], cats [23] and buffaloes [24], showed that artificial collapse of the blastocoel cavity by BF removal improves cryotolerance and embryo viability after vitrification/warming. Some authors claim that this manipulation is not far from resembling to the physiological situation, because spontaneous collapse-expansion cycles occurs during expansion and hatching of blastocysts [16], probably to diminish the thickness of the zona pellucida and assist in hatching [25].

Although knowledge of the underlying mechanisms regulating natural expansion-collapse events is still scarce, growing evidence on the function of blastocoel cavity and the composition of BF has been published in cattle [26–28]. While it is known that the BF is formed by water and nutrients transported through the TE selective barrier, it has been recently suggested that the BF could directly support the development of bovine blastocysts and play a key role in their preimplantation [27], rather than merely providing a compartment for cell migration. Interestingly, apart from metabolites [28] and proteins [29], cell-free DNA (cfDNA) is found in the BF of humans [30–32] and horses [21]. After being analysed for preimplantation genetic testing (PGT), the cfDNA contained in the BF of these human and equine blastocysts can provide accurate genetic information about the embryo, pointing to a genomic origin of the DNA, which would probably come from apoptotic cells. To the best of the authors' knowledge, nevertheless, whether cfDNA is present and can be isolated from the BF of cattle embryos has not been investigated.

In this study, it was hypothesised that the use of BF aspiration and artificial collapse before vitrification could be an effective technique for sexing and cryopreserving bovine IVP blastocysts by providing a practical and reliable source of cfDNA and improving post-warming outcomes. Thus, the objective of this study was to test the BF as a source of cfDNA and to compare it to the TE biopsy in terms of (a) sexing efficiency/accuracy, and its effect on (b) embryo re-expansion and hatching and (c) gene expression following vitrification/warming.

2. Materials and methods

2.1. Chemicals and suppliers

Except where otherwise noted, all chemicals and reagents were supplied from Sigma-Aldrich (Merck, MA, USA).

2.2. Experimental design

In the first set of experiments, IVP Grade 1 D7 expanded blastocysts were selected, and randomly assigned to either group: VIT-Collapsed, embryos were artificially collapsed before vitrification to obtain a BF sample; or VIT-Biopsied, embryos were biopsied before vitrification to obtain a TE biopsy sample. Both BF and TE samples were stored at −80 °C until further examination. Collapsed or biopsied IVP embryos were vitrified/warmed and only surviving blastocysts at 24 h post-warming were individually stored −80 °C for subsequent analysis. All three specimens, BF samples, TE biopsies, and remaining blastocysts, were analysed separately for sex determination by identifying the presence of *BRY4a* and *SAT1* genes in nuclear DNA through conventional PCR. This experiment was conducted independently in three replicates. Here, amplification efficiency (proportion of samples with *BRY4a* and/or *SAT1* amplification out of the total number of samples analysed) and sexing accuracy (proportion of samples that coincided in the sex diagnosis with their corresponding blastocysts out of the number of samples successfully amplified), as well as gender ratio (proportion of samples of each sex out of the total number of accurate samples) were assessed.

In the second series of experiments, IVP Grade 1 D7 expanded blastocysts were selected, and randomly allocated to one of the following groups: 1) VIT-Control, intact embryos that were vitrified/warmed and cultured individually for 24 h once warmed; 2) VIT-Collapsed, embryos that were artificially collapsed to harvest the BF sample, vitrified/warmed and cultured individually for 24 h once warmed; 3) VIT-Biopsied, embryos that were biopsied to obtain the TE biopsy sample, vitrified/warmed and cultured individually for 24 h once warmed. The Control group consisted of fresh non-vitrified intact embryos individually cultured for 24 h. Additionally, the Control-Collapsed and Control-Biopsied groups consisted of fresh non-vitrified embryos that were artificially collapsed and biopsied, respectively, both individually cultured for 24 h. The survival of vitrified blastocysts was determined as re-expansion rates after 3 h and 24 h of recovery in *in vitro* culture medium, while hatching rates were assessed at 24 h post-warming. This experiment was conducted independently in seven replicates. Surviving embryos from the three vitrified groups and the fresh Control group were classified as expanded or hatching/hatched and stored at −80 °C for measuring, by reverse transcription real-time PCR

(RT-qPCR), the relative expression of six genes related to apoptosis (*BAX*, *BCL2*), ion flux (Na^+/K^+ ATPase; *ATP1B1*), water transport (*AQP3*), lipid metabolism (*SCD2*), and oxidative stress (*GPX1*). Four independent replicates were examined.

2.3. In vitro production of bovine embryos

In vitro production of bovine embryos was performed as previously described by Martinez-Rodero et al. [33], with slight modifications.

For *in vitro* maturation (IVM) of oocytes, ovaries from apparently healthy postpubertal heifers (12–18 months) were collected from a local abattoir (Escorxador Sabadell S.L., Sabadell, Spain) from October to June and transported to the laboratory in saline solution (0.9 % NaCl) at 35–37 °C. Follicles from 3 to 8 mm in diameter were aspirated using an 18 g needle to obtain cumulus-oocyte complexes (COCs). COCs with three or more cumulus cells layers and homogeneous cytoplasm were selected for IVM and washed thrice in modified Phosphate-Buffered Saline (PBS) enriched with 36 mg/mL pyruvate, 50 mg/mL gentamicin, and 0.5 mg/mL bovine serum albumin (BSA). An average of 53 ± 11 ovaries and 371 ± 15 COCs (7 COCs/ovary) were obtained in each replicate (mean ± standard deviation). Selected COCs were moved in groups of 40–50 COCs to 500-μL wells of IVM medium and cultured for 24 h at 38.5 °C in a 5 % CO_2 humidified atmosphere. IVM medium was composed of bicarbonate-buffered tissue culture medium 199 (TCM-199) supplemented with 10 % (v/v) foetal bovine serum (FBS), 10 ng/mL epidermal growth factor and 50 mg/mL gentamicin.

For *in vitro* fertilization (IVF) of oocytes, frozen-thawed sperm from a proven fertility Asturian bull (ASEAVA, Asturias, Spain) were centrifuged at 300 g for 10 min through a 1 mL 40 %-1 mL 80 % density gradient (BoviPure diluted in Bovidilute; Nicadon International AB, Göthenburg, Sweden), resuspended in 3 mL of Boviwash (Nicadon International AB, Göthenburg, Sweden) and pelleted by centrifugation at 300*g* for 5 min. After counting in a Neubauer chamber, the resulting good morphology and high motility sperm were diluted in the corresponding volume of IVF medium to obtain a concentration of $2x10^6$ sperm/mL. Groups of 40–50 COCs were transferred from IVM medium to 250-μL well of IVF medium, inseminated with a final concentration of $1x10^6$ sperm/mL by adding 250 mL of sperm suspension, and co-incubated for 18 h at 38.5 °C in a 5 % CO_2 humidified atmosphere. The IVF medium consisted of 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA, and 10 mg/mL heparin–sodium salt.

For *in vitro* culture (IVC) of embryos, presumptive zygotes were pipetted and denuded in 500-μL well of PBS after 18 h post-insemination (hpi). Then, they were washed and placed in 25 μL drops (1 μL/embryo) of IVC media covered with 3.5–4 mL of Nidoil (Nicadon International AB, Göthenburg, Sweden) and cultured for 168 hpi at 38.5 °C in a 5 % CO_2, 5 % O_2 humidified atmosphere. The IVC medium was based on synthetic oviductal fluid (SOF; Caisson Labs, UT, USA) supplemented with 88.6 μg/mL sodium pyruvate, 2 % (v/v) non-essential amino acids, 1 % (v/v) essential amino acids, 0.96 μg/mL BSA, 2 % (v/v) FBS and 0.5 % gentamicin. After 7 days in culture, Grade 1 expanded blastocysts [34] were randomly assigned to experimental treatments (see Experimental Design). To assess the appearance of blastocysts selected for these experiments, Supplementary Fig. 1 is provided.

2.4. Obtaining blastocyst samples for sex determination

All steps were performed by the same operator wearing a lab coat, nonpowered sterile gloves, and mask. Surfaces, equipment, and automatic pipettes were previously cleaned with 70 % ethanol. ICSI pipettes were replaced after each BF aspiration. After each TE biopsy, microblades were cleaned with 70 % ethanol. The tips used to prepare drops and handle BF and TE biopsy samples were DNAse and RNAse free, with filter, sterile, and discarded after use.

2.4.1. BF aspiration and collection by blastocoel cavity collapse

The BF aspiration protocol was as described elsewhere [21], with some modifications. Each D7 blastocyst at the expanded stage was placed in a 25-μL drop of Holding Medium (HM; Hepes-buffered TCM 199 with 20 % (v/v) FBS) covered with 4 mL of mineral oil on an inverted microscope (Zeiss Axio Vert A1, Oberkochen, Germany) equipped with a 38.5 °C heated stage (Okolab S.r.l., Pozzuoli, Italy) and an Eppendorf micromanipulator system (Eppendorf Corporate, Hamburg, Germany). By suction through a holding pipette (20 μm inner diameter; MPHL-35, LifeGlobal group, CT, USA), each blastocyst was held with the inner cell mass (ICM) placed at the 12 or 9 o'clock position. The ICSI pipette was immersed in the HM drop and its content was expelled until seeing an air bubble. Once the zona pellucida was punctured with a bevelled spiked ICSI pipette (4.5–5.0 μm inner diameter; MPHL-35, LifeGlobal group, CT, USA), the BF was aspirated until the blastocoel cavity was collapsed but before any cell content could be accidently suctioned. Occasionally, collapsing of the blastocoel occurred partially or incompletely, because the elastic plasma membrane of trophectoderm cells adhered to the puncture opening. In those cases, puncturing was performed again at a different site of the trophectoderm until complete collapsing of blastocoel was achieved. Fig. 1 shows representative images of the artificial collapse procedure.

Thereafter, the BF contained in the ICSI pipette was discharged to a 1-μL drop of DEPC-treated water (Thermo Fisher Scientific, MA, USA) until seeing an air bubble. The whole volume of this drop was collected, transferred to a 0.20-mL DNase-free tube, snap-frozen in liquid nitrogen, and stored at −80 °C for further sex determination. BF samples containing any cells were discarded for this study. Each collapsed embryo was carefully washed three times in HM for its immediate individual vitrification.

2.4.2. TE conventional biopsy by microblade cutting

The TE conventional biopsy was carried out as described by González-Rodríguez et al. [12], with slight modifications. Each D7 expanded blastocyst was placed in a 50-μL drop of HM and covered with 4 mL of mineral oil on a 38.5 °C heated stage (Okolab S.r.l., Pozzuoli, Italy) and an inverted Zeiss microscope-Eppendorf micromanipulator system (Zeiss Axio Vert A1, Oberkochen, Germany; Eppendorf Corporate, Hamburg, Germany). The base of each dish was previously scratched to limit the movement of the embryo during manipulation. A microsurgical blade (Stab 6200, Sidapharm, Thessaloniki, Greece) was adjusted to the right-side micromanipulator to rotate the blastocyst until the ICM was visible, and to press a portion of the TE cells, at 180° clockwise away from the ICM, against the bottom of the dish. Then, the dish was gently glided until a small portion of the embryo (5–15 TE cells) was cut off.

The biopsied cells were recovered, washed in DEPC-treated water (Thermo Fisher Scientific, MA, USA), transferred to a 0.20-mL DNase-free tube, snap-frozen in liquid nitrogen, and stored at −80 °C for further sex determination. Each biopsied embryo was carefully washed three times in HM for its immediate individual vitrification.

2.5. Embryo vitrification and warming

D7 grade 1 expanded blastocysts from each experimental group (see Experimental Design) were vitrified/warmed following the short-equilibration protocol of the Cryotop® method, as described by Martinez-Rodero et al. [15]. Fresh D7 grade 1 non-vitrified blastocysts were cultured individually for 24 additional hours and served as fresh non-vitrified controls (see Experimental Design).

2.5.1. Vitrification protocol

Blastocysts were immersed in equilibration solution (ES; HM with 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) dimethyl sulfoxide (DMSO)). After 3 min, blastocysts were transferred to the vitrification solution (VS; HM containing 15 % (v/v) EG, 15 % (v/v) DMSO, and 0.5

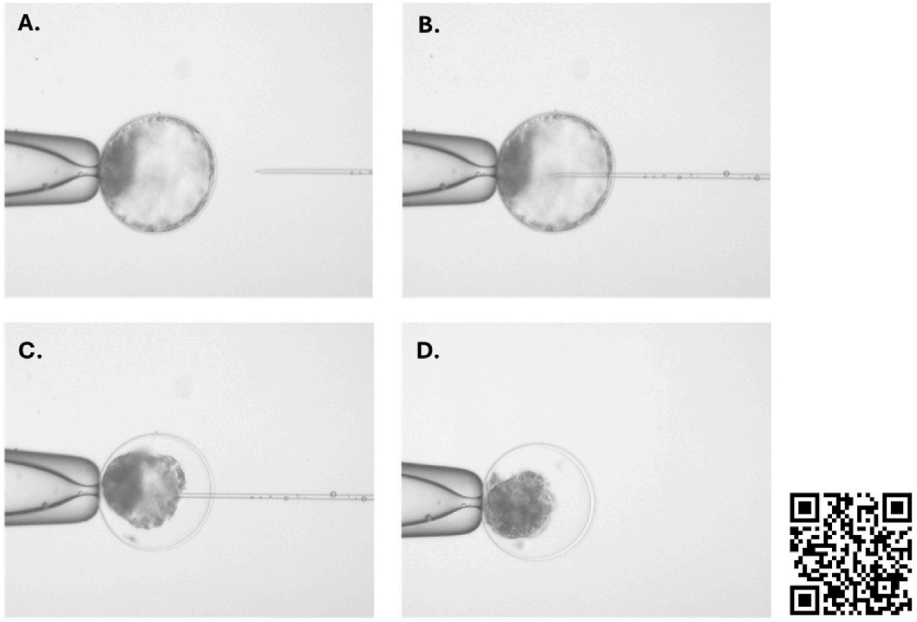

Fig. 1. Collapse of the blastocoel cavity by aspiration of blastocoel fluid using an ICSI pipette. (A) Intact blastocyst held by the holding pipette with the inner cell mass (ICM) placed at the 9 o'clock position. (B) Blastocyst punctured by the ICSI pipette, avoiding contact with the ICM. (C) Blastocyst with its blastocoel cavity partially collapsed after gentle aspiration of the BF with the ICSI pipette. (D) Collapsed blastocyst after complete BF aspiration. QR code shows a video of the whole procedure.

M sucrose) for 30–40 s. Then, each blastocyst was placed onto the Cryotop® sheet with minimal volume, and the excess of VS was removed leaving only a thin layer of the solution covering the blastocysts. Immediately after, the Cryotop® was plunged into liquid nitrogen and inserted into the straw cap. The whole procedure from the first contact of the blastocyst with VS to the Cryotop® plunging into liquid nitrogen took less than 1 min. The loaded devices were stored in liquid nitrogen.

2.5.2. Warming protocol

Blastocyst warming started by quickly immersing the Cryotop® sheet tip in HM with 1 M sucrose. Next, the blastocyst was transferred and incubated in HM supplemented with 0.5 M sucrose for 3 min and then in HM for 5 min. Finally, each blastocyst was individually cultured in IVC medium (20 μL/drop) for 24 h at 38.5 °C in a 5 % CO_2, 5 % O_2 humidified atmosphere. Blastocysts were evaluated under a stereomicroscope to assess their re-expansion rates (3 h and 24 h post-warming) and their hatching rates (24 h post-warming).

Collapsed/biopsied blastocysts that survived after 24 h of warming were washed thrice in pronase to remove possible sperm cells attached to the zona pellucida, snap frozen in liquid nitrogen, and individually stored at −80 °C until their further sex determination.

For gene expression analysis, groups (from 2 to 5) of surviving blastocysts were washed three times in Dulbecco's PBS with 0.01 % (w/v) polyvinyl alcohol at 38.5 °C, pipetted within minimal volume into 1.5 mL tubes, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction and RT-qPCR analysis.

2.6. Embryo sexing by PCR

Sex determination was based on the PCR protocol described by Bermejo et al. [35], with some modifications.

BF samples underwent Whole Genome Amplification (WGA) according to manufacturer's instructions (REPLI-g single cell kit, Qiagen, MD, USA). Briefly, samples were incubated at room temperature with 2.5 μL of Denaturation buffer. After 3 min, the denaturation reaction was stopped by adding 0.5 μL of Neutralization buffer. Then, 40 μL of the master mix was added and incubated with the BF samples for 16 h at 30 °C. The amplification reaction was stopped by raising the temperature to 65 °C for 3 min. The amplified DNA was kept at 4 °C until further processing. TE biopsies and blastocysts were digested with 8 μL of 100 μg/mL proteinase K at 55 °C for 2 h to increase PCR efficiency. After inactivation of the enzyme at 95 °C for 10 min, digested DNA was kept at 4 °C until further analysis.

BF samples, TE biopsies, and blastocysts were analysed by conventional PCR. Two sets of primers were used to determine embryo sex: Y-chromosome-specific sequence in males (*BRY4a*) and bovine-specific satellite sequence primers (*SAT1*), as a marker (see Table 1) [35]. Because of the number of repetitions of these sequences, this is one of the best strategies to sex bovine embryos in a single PCR [36]. Samples were thawed at room temperature and centrifuged at 8000 g for 30 s before being mixed with PCR reagents. Following the fabricant indications, tubes for the PCR reactions contained 25 μL PCR Master Mix (2x) (ThermoFisher, MA, USA), 2 μL *BRY4a* forward and reverse primers (1 μM; Fisher Scientific, MA, USA), 2 μL *SAT1* forward and reverse primers (1 μM; Fisher Scientific, MA, USA), 16 μL DEPC-treated water and 1 μL sample DNA, with a total volume of 50 μL. The PCR reaction consisted of a first cycle of 3 min at 94 °C, 40 s at 60 °C and 15 s at 72 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at 63 °C, 15 s at 72 °C and 5 min at 72 °C, in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany). PCR products were visualised on a 2 % (w/v) agarose gel stained with 0.003 % (v/v) SafeView™ Plus (Applied Biological Materials, British Columbia, Canada) after a run for 1 h at 80 V. Initially, two controls to

Table 1
Two sets of primers were used for conventional PCR-based sex determination [35].

Symbol	Primer Sequences (5′-3′)		Amplicon Size
BRY4a (Repetitive sequence specific to Y-chromosomal DNA sequence)	Fw:	CTCAGCAAAGCACACCAGAC	300 bp
	Rv:	GAACTTTCAAGCAGCTGAGGC	
SAT1 (spermidine/spermine N1-acetyltransferase 1)	Fw:	TGGAAGCAAAGAACCCCGCT	216 bp
	Rv:	TCGTGAGAAACCGCACACTG	

Fw: Forward; Rv: Reverse.

discard exogenous DNA contamination of tested samples were included: (1) DEPC-water where BF and TE biopsy samples were collected, and (2) blank medium plus mineral oil used for IVC culture. A negative control (no template) and two positive controls for male (testis genomic DNA) and female (ovaries genomic DNA) were included in every run. Gels were examined in a UV scanner for the 300-bp band of *BRY4a* and the 216-bp band of the *SAT1*. Samples showing both *BRY4a/SAT* bands were considered male; samples with only the *SAT1* band were assigned as female, and samples showing no band were recorded as non-amplified. To confirm the results obtained from conventional PCR agarose gels, amplified products were also analysed through a High Sensitivity DNA Assay using a Bioanalyzer (2100 Bioanalyzer Instrument; Agilent Technologies, CA, USA).

2.7. Gene expression analysis

The procedures used for RNA extraction and RT-qPCR have been described elsewhere [37].

First, total RNA was extracted from blastocyst pools (4 pools of 5 expanded blastocysts and 4 pools of 2–3 hatching/hatched blastocysts) according to the manufacturer's instructions (RNeasy Kit; Qiagen, MD, USA) with slight modifications.

After extraction, High-Capacity cDNA Reverse Transcription kit (Thermo Fisher, MA, USA) was used to prepare the RT master mix, which was added in proportion 1:1 (v/v) to the extracted RNA (15–17 μL). The RT reaction was run in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany) for 10 min at 25 °C, followed by 120 min at 37 °C to allow the reverse transcription of mRNA and for 5 min at 85 °C to denature the MultiScribe™ reverse transcriptase.

The resulting cDNA was used to quantify the relative abundance of mRNA transcripts of six genes by qPCR (*BAX*, *BCL-2*, *ATP1B1*, *AQP3*, *SCD2*, and *GPX1*). *PPIA* and *H3F3* were set as housekeeping genes, as they were previously tested for stability in bovine blastocysts [38]. In brief, 1 μL cDNA template was mixed with the qPCR Master Mix: 10 μL of Fast SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA), 1.5 μL of each primer (500 nM; Thermo Fisher Scientific, MA, USA; Table 2), and DEPC-treated water (Thermo Fisher Scientific, MA, USA) to a final volume of 20 μL. The RT-qPCR reaction 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 s, an annealing step for 1 min at 60 °C (primers annealing temperature) and a final extension step at 72 °C for 40 s, using a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Two technical replicates from each biological replicate per individual gene, and negative controls for the primers were included in each reaction. Before analysing samples, a calibration curve was run for each primer to check a minimum amplification efficiency of 80 %; the melting curve of each amplified PCR product was verified and run in agarose gel electrophoresis (2 % agarose gel containing 0.1 μg/mL SafeView™ Plus; Applied Biological Materials, British Columbia, Canada) for 1 h at 80 V.

The relative expression of the six candidate genes (*BAX*, *BCL2*, *ATP1B1*, *AQP3*, *SCD2*, and *GPX1*) in vitrified/warmed surviving blastocysts was quantified using the comparative threshold cycle (Ct) method [39]. To determine the Ct for each sample, fluorescence data were acquired after each elongation step. Following the comparative Ct method, the mean of housekeeping (HK) genes *PPIA* and *H3F3A* Ct values for each sample were subtracted from the Ct value separately for each replicate, target gene and blastocysts stage (Expanded or Hatching/ed) to calculate the ΔCt value. Then, each ΔCt value was subtracted from the ΔCt value for the fresh Control group to determine ΔΔCt scores. Fold differences in relative transcript abundances were calculated for target genes assuming 100 % amplification efficiency using the formula $2^{-(\Delta\Delta Ct)}$. Negative controls for the template were not amplified or returned a Ct 10 points higher than the average Ct for the genes amplified in samples. Used primer sequences, their GenBank accession numbers, and amplicon sizes are provided in Table 2.

2.8. Statistical analysis

To perform all statistical tests and graphs, the software GraphPad Prism 8 (GraphPad Software, CA, USA) and SPSS v. 26 (IBM, NY, USA) for Windows were used. Data were first checked for normality using the Shapiro-Wilk's test and for homogeneity of variances using the Levene test.

After checking data normality and homoscedasticity, survival outcomes and relative transcript abundances were compared between treatment groups by analysis of variance (ANOVA) followed by Tukey's test for pair-wise comparisons. Amplification efficiency, sexing accuracy and gender ratio were compared between different sources of DNA by Chi-square test. Gender ratio was also compared with the natural ratio (50 %:50 %) by Chi-square test.

The mean ± standard error of the mean (SEM) is used to express data. Significance was set at $p \leq 0.05$ (two-tailed).

Table 2
Primers used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (NCBI, National Centre for Biotechnology Information).

Symbol	Primer Sequences (5′-3′)		Amplicon Size	GenBank Accession nº
BAX (BCL2 associated X apoptosis regulator)	Fw:	ACCAAGAAGCTGAGCGAGTG	116 bp	NM_173894.1
	Rv:	CGGAAAAAGACCTCTCGGGG		
BCL2 (BCL2 apoptosis regulator)	Fw:	GGCCCCTGTTTGATTTCTCCT	99 bp	NM_001166486.1
	Rv:	ACTTATGGCCCAGATAGGCAC		
ATP1B1 (ATPase Na+/K+ transporting subunit β1)	Fw:	GCCCACATATCAGGACCGAG	90 bp	NM_001035334.1
	Rv:	GGATCGTTAGGACGAAAGGCA		
AQP3 (Aquaporin 3)	Fw:	GTACGTGTGCGTGGTTTTCC	72 bp	NM_001079794.1
	Rv:	CCCAACTCCACCGACAGAAT		
SCD2 (Stearoyl-Coenzyme A desaturase)	Fw:	CCGCCCTTATGACAAGACCA	87 bp	NM_173959.4
	Rv:	TGGTGGTAGTTGTGGAAGCC		
GPX1 (Glutathione Peroxidase 1)	Fw:	CTGAAGTACGTCCGACCAGG	153 bp	NM_174076.3
	Rv:	GTCGGTCATGAGAGCAGTGG		
PPIA (Peptidylprolyl isomerase A)	Fw:	CATACAGGTCCTGGCATCTTGTCC	108 bp	NM_178320.2
	Rv:	CACGTGCTTGCCATCCAACC		
H3F3A (H3.3 histone A)	Fw:	CATGGCTCGTACAAAGCAGA	136 bp	NM_001014389.2
	Rv:	ACCAGGCCTGTAACGATGAG		

Fw: Forward; Rv: Reverse.

3. Results

3.1. BF as a source of cfDNA produces sexing results comparable to microblade TE biopsy

Twenty-five collapsed and twenty-nine biopsied blastocysts that survived vitrification/warming and their corresponding BF and TE biopsy samples were analysed for sex determination. Fig. 2 shows a representative gel electrophoresis of products amplified using DNA extracted from BF, TE and blastocysts using conventional PCR-based sex determination and High Sensitivity DNA Assay. Three different situations were observed. (1) No amplification: there was a lack of amplification in BF, TE biopsy, or blastocyst samples; (2) Amplification and accuracy: the bands observed in the BF or TE biopsy matched with those amplified in the blastocysts; and (3) Amplification and inaccuracy: the bands observed in the BF or TE biopsy did not match with those amplified in the blastocysts. Table 3 shows the percentages of amplification efficiency, sexing accuracy, and gender ratios obtained from both DNA sources (BF or TE biopsy).

There were no significant differences ($p > 0.05$) in the amplification efficiency or sexing accuracy between the two DNA sources. When the gender ratio in all samples was examined, a greater percentage (though not statistically significant ($p = 0.09$) of males than females was observed.

In detail, from the 25 BF samples analysed, six failed to amplify sexing primers *BRY4a* or *SAT1* (Situation 1); 15 gave positive amplification and accurate sex diagnosis (Situation 2); and four yielded positive amplification but failed to correctly diagnose genetic sex (Situation 3). These four BF samples wrongly indicated the blastocyst was a female. Out of the 29 studied embryos, six TE biopsy samples resulted in no amplification (Situation 1), 19 samples ended in amplification and accuracy (situation 2) and four samples gave amplification and inaccuracy (Situation 3). In the latter case, three TE biopsy samples wrongly indicated the blastocyst was a female and one that it was a male.

The High Sensitivity DNA Assay revealed the presence of DNA fragments in 88.00 % of BF samples (after the previous WGA) and 100 % of TE Biopsy samples (no previous WGA). Moreover, the absence of specific amplicons from negative controls (no template control, DEPC-water, blank IVC medium plus mineral oil) discarded DNA environmental contamination in the tested samples.

Table 3
Amplification efficiency, sexing accuracy, and gender ratio for the different sources of DNA. Data are expressed as percentages.

	Amplification efficiency (%)	Sexing accuracy (%)	Gender ratio (%)	
Source of DNA			*Male*	*Female*
Blastocoel Fluid	76.00 (19/25)	78.90 (15/19)	66.70 (10/15)	33.30 (5/15)
TE Biopsy	79.30 (23/29)	82.60 (19/23)	63.20 (12/19)	36.80 (7/19)
p-value	0.77	0.68	0.52	0.43

Amplification efficiency: proportion of samples with *BRY4a* and/or *SAT* amplification out of the total number of samples analysed; sexing accuracy: proportion of samples that coincided in the sex diagnosis with their corresponding blastocysts out of the number of samples successfully amplified; Gender ratio: proportion of samples of each sex out of the total number of accurate samples.
Male: samples where *BRY4a* and *SAT1* genes were amplified; Female: samples where only the *SAT1* gene was amplified.
p-values as a result of the Chi-square test. Amplification efficiency, sexing accuracy and gender ratio were compared between the different sources of DNA. Gender ratio was also compared to the natural ratio (i.e., 50 %:50 %).
Six samples where neither the BF/TE biopsy nor its corresponding blastocyst were amplified were discarded from the analysis.

3.2. Blastocyst artificial collapse before vitrification improves post-warming re-expansion and hatching rates of D7 IVP embryos

Table 4 shows post-warming re-expansion and hatching rates of vitrified/warmed D7 expanded blastocysts. At 3 h post-warming, the Control-Collapsed group showed similar re-expansion rates to the Control group, and significantly higher than those observed in the other treatment groups.The lowest rates were found in the blastocysts that underwent biopsy, in both the Control-Biopsied and VIT-Biopsied groups ($p < 0.05$). At 24 h post-warming, neither the collapse nor the biopsy had any impact on the re-expansion rates of fresh non-vitrified embryos compared to the control. However, vitrified embryos and biopsied vitrified embryos exhibited lower re-expansion rates ($p < 0.05$),

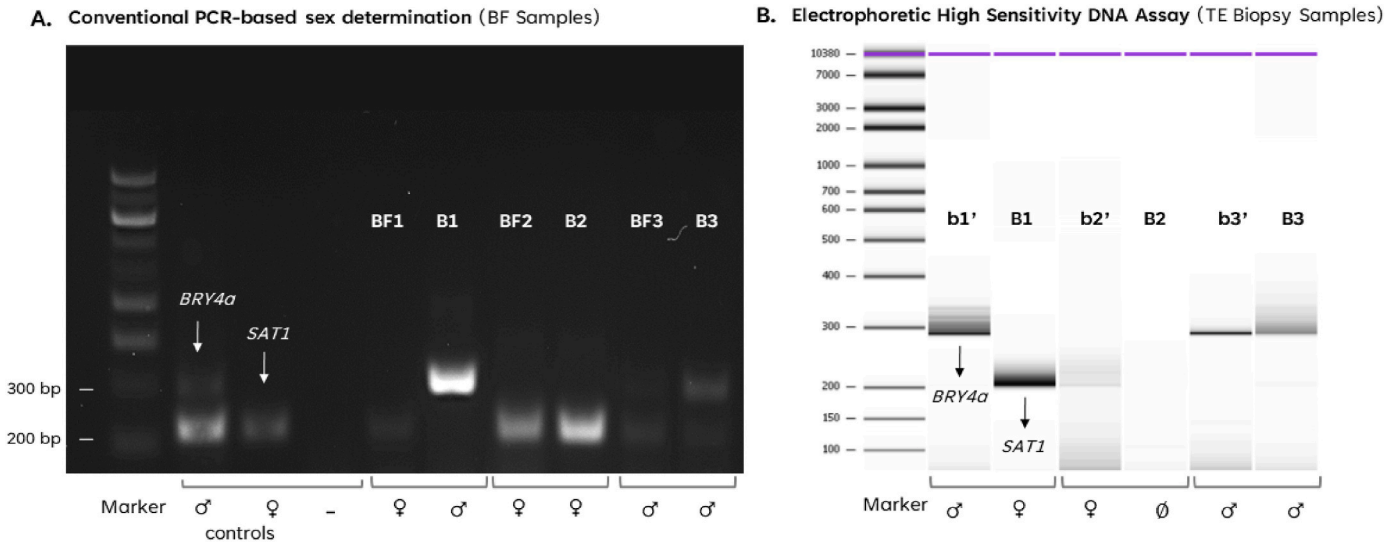

Fig. 2. Representative image of **(A)** conventional PCR-based sex determination showing some results derived from blastocoel fluid (BF) samples, and **(B)** electrophoretic High Sensitivity DNA Assay (Bioanalyzer) showing results from some trophectoderm (TE) biopsy samples.
Marker: molecular weight indicator (bp); ♀: Female; ♂: Male; ∅: no amplification; BF: Blastocoel Fluid; b': biopsy; B: Corresponding blastocyst; *BRY4a*; Repetitive sequence specific of Y-chromosomal DNA sequence; *SAT1*: spermidine/spermine N1-acetyltransferase 1.
♀ control: ovarian tissue sample; ♂ control: testicular tissue sample. – control: non-template control. (1) No amplification: b'2 and B2; (2) Amplification and accuracy: BF2 and B2, BF3 and B3, b3′ and B3; (3) Amplification and inaccuracy: BF1 and B, b1′ and B1

Table 4
Post-warming re-expansion and hatching rates of D7 expanded blastocysts after each vitrification treatment. Data are shown as mean ± SEM.

Treatment	Post-warming			
	nº of embryos	Re-expansion rate (%) (3 h)	Re-expansion rate (%) (24 h)	Hatching rate (%) (24h)
Control	132	100.00 ± 0^{a}	100.00 ± 0^{a}	41.67 ± 4.23^{ac}
Control-Collapsed	58	94.00 ± 3.00^{a}	96.00 ± 4.00^{ab}	30.48 ± 4.36^{a}
Control-Biopsied	49	29.07 ± 3.24^{b}	93.75 ± 12.50^{ab}	75.97 ± 9.24^{b}
VIT-Control	120	59.79 ± 2.98^{c}	81.36 ± 3.52^{b}	30.46 ± 1.47^{a}
VIT-Collapsed	69	63.04 ± 6.78^{c}	91.83 ± 3.74^{ab}	46.12 ± 2.42^{c}
VIT-Biopsied	105	23.45 ± 3.65^{b}	57.86 ± 2.94^{c}	38.37 ± 2.19^{a}

^{a,b,c} Values within columns with different superscripts indicate significant differences ($P < 0.05$) between groups according to ANOVA and Tukey's test. Re-expansion rate: proportion of blastocysts that were able to re-expand from the total number of warmed blastocysts (including those hatching/ed); Hatching rate: proportion of hatching/hatched blastocysts from the total number of warmed blastocysts. Control: fresh expanded blastocysts individually cultured for 24 h; Control-Collapsed: fresh embryos artificially collapsed and individually cultured for 24 h; Control-Biopsied: fresh biopsied embryos individually cultured for 24 h. VIT-Control: intact blastocysts vitrified and individually cultured for 24 h after warming; VIT-Collapsed: collapsed blastocysts vitrified and individually cultured for 24 h after warming; VIT-Biopsied: biopsied blastocysts vitrified and individually cultured for 24 h after warming.

whereas collapsed vitrified embryos showed re-expansion rates similar to those observed in the fresh non-vitrified groups.

Biopsied blastocysts showed the highest hatching rate at 24 h post-warming when compared to other treatment groups ($p < 0.05$). Blastocysts that underwent vitrification following blastocoel collapse exhibited significantly greater hatching rates compared to both their fresh counterparts and those blastocysts that were only vitrified or vitrified after biopsy. None of the vitrification treatments, nevertheless, showed a significant difference from the control fresh group.

3.3. Gene expression

Thirty-eight collapsed, 32 biopsied and 40 intact blastocysts that survived vitrification/warming and 40 fresh non-vitrified blastocysts were analysed by RT-qPCR to determine the mRNA relative abundance of six genes related to apoptosis (*BAX*, *BCL2*) and its ratio *(BAX/BLC2)*, Na^{+}/K^{+} ATPases (*ATP1B1*), water movement (*AQP3*), lipid metabolism (*SCD2*), and oxidative stress (*GPX1*). The results of this analysis are illustrated in Fig. 3. Melting and amplification curves for each gene studied are included in Supplementary Fig. 2.

The levels of proapoptotic gene *BAX* were higher ($p < 0.05$) in expanded blastocysts derived from the VIT-Biopsied group than in those of the Fresh Control group. Hatched blastocysts derived from the VIT-Collapsed group showed lower ($p < 0.05$) relative abundance of the *BAX* transcript than those derived from the VIT-Biopsied treatment. Contrarily, transcript abundance of *BCL2* (an antiapoptotic gene) was higher ($p < 0.05$) in VIT-Collapsed hatched blastocysts than in the VIT-Biopsied hatching ones. In spite of this, no significant differences ($p > 0.05$) between groups were found in the *BAX/BLC2* ratio. The expression of the Na^{+}/K^{+}-ATPase pump gene *ATP1B1* was higher both in the expanded and hatched blastocysts derived from the VIT-Biopsied group than in those derived from the other groups (Fresh Control, VIT-Collapsed). The *AQP3* gene was upregulated ($p < 0.05$) in hatched blastocysts derived from VIT-Biopsied blastocysts when compared to those of the Fresh Control group. No differences in the relative abundance of *SCD2* and *GPX1* were observed between groups.

4. Discussion

Blastocoel fluid aspiration and artificial collapse prior to vitrification may offer an effective method for sexing and cryopreserving bovine IVP blastocysts. The current work hypothesised that the cfDNA present in BF could be collected from fully expanded IVP blastocysts, thus offering a promising less invasive methodology for bovine embryo sexing. Furthermore, the artificial collapse induced in blastocysts during BF aspiration prior to vitrification could be beneficial for their cryotolerance and improve the survival outcomes obtained with microblade TE biopsy.

In the present study, cfDNA of BF samples obtained from IVP bovine blastocysts was, for the first time, successfully amplified through WGA and quantified by conventional PCR, with an amplification efficiency and sexing accuracy (76.0 % and 78.9 %, respectively) comparable to those obtained with a TE biopsy (79.3 % and 82.6 %, respectively). Similar results to ours were observed in two separate studies in horses [21] and humans [30]. Whereas BF sexing in humans produced results lightly inferior than ours (65.4 %, 17/26 accurately sexed embryos) [30], BF sexing in equine species [21] demonstrated that the sex of 84 % (11/13) *in-vivo* derived and that of 80 % (4/5) IVP embryos could be diagnosed using the blastocoel fluid as a DNA source. Differences in accuracy between the equine study and ours could be attributed to the greater number of samples examined here or to the large blastocoel size and BF volume of horse blastocysts.

While the High Sensitivity DNA Assay detected DNA fragments in 88.0 % of BF samples, there is not much room for improvement in amplification efficiency. Several challenges stand in the way of this improvement: firstly, it would be challenging to increase cfDNA concentration in BF samples collected in volumes smaller than 1 μL, especially under mineral oil; secondly, the inclusion of the WGA step has already been implemented for sample enrichment; and thirdly, targeting multicopy genes like *BRY4a* is considered a suitable approach for determining embryo sex, particularly in samples with low DNA quantities [21,30]. The limitation in *BRY4a/SAT* amplification may be due to some degree of cfDNA degradation, as could be expected given its presumptive apoptotic origin. It is extensively demonstrated that *in-vitro* fertilised and cultured bovine blastocysts usually have fragmented nuclei in both the TE and ICM cells [15,33,40]. As a result, fragmented DNA may be released and detected in the blastocoel cavity. It is still unknown whether the cfDNA released to BF comes from apoptotic cells with normal or abnormal karyotypes or only from "healthy" cells of TE or ICM [41].

The embryo sex of four BF samples was misdiagnosed. The cause or origin of this failure as well as how the accuracy should be improved deserves further attention. Misdiagnosis of male embryos as females has been previously observed in other studies that used both cfDNA (12.5 % [42]) and the whole embryo (14.0 % [43]) as a DNA source for sexing. Amelogenin (*AMEL* gene) is a so-called built-in technique where a single pair of primers amplify a Y-chromosome-specific sequence and a second by-product [44]. Using the whole embryo lysate as a source of DNA, Trigal et al. [43] demonstrated that the use of *AMEL* gene produces more accurate results than the two pairs of *BRY4a/SAT1* primers, and this because using the same primers to identify male and female sequences eliminates errors caused by specific primer amplification efficiency. In preliminary studies (data not shown), we attempted to sex blastocysts from BF samples using *AMEL* primers but amplification efficiency was very low (2/10 samples successfully amplified). This finding was previously reported by Herrera et al. [21], who found that a multi-copy gene, the *TSPY* gene, had greater amplification efficiency than the single-copy gene *AMEL*.

While *in vitro* culture conditions play a significant role in the low cryotolerance of IVP embryos [11], many other factors influence vitrification results, including technical and biological aspects. Although expanded blastocysts produce better survival outcomes than other embryo development stages [45,46], the presence of water in the blastocoel

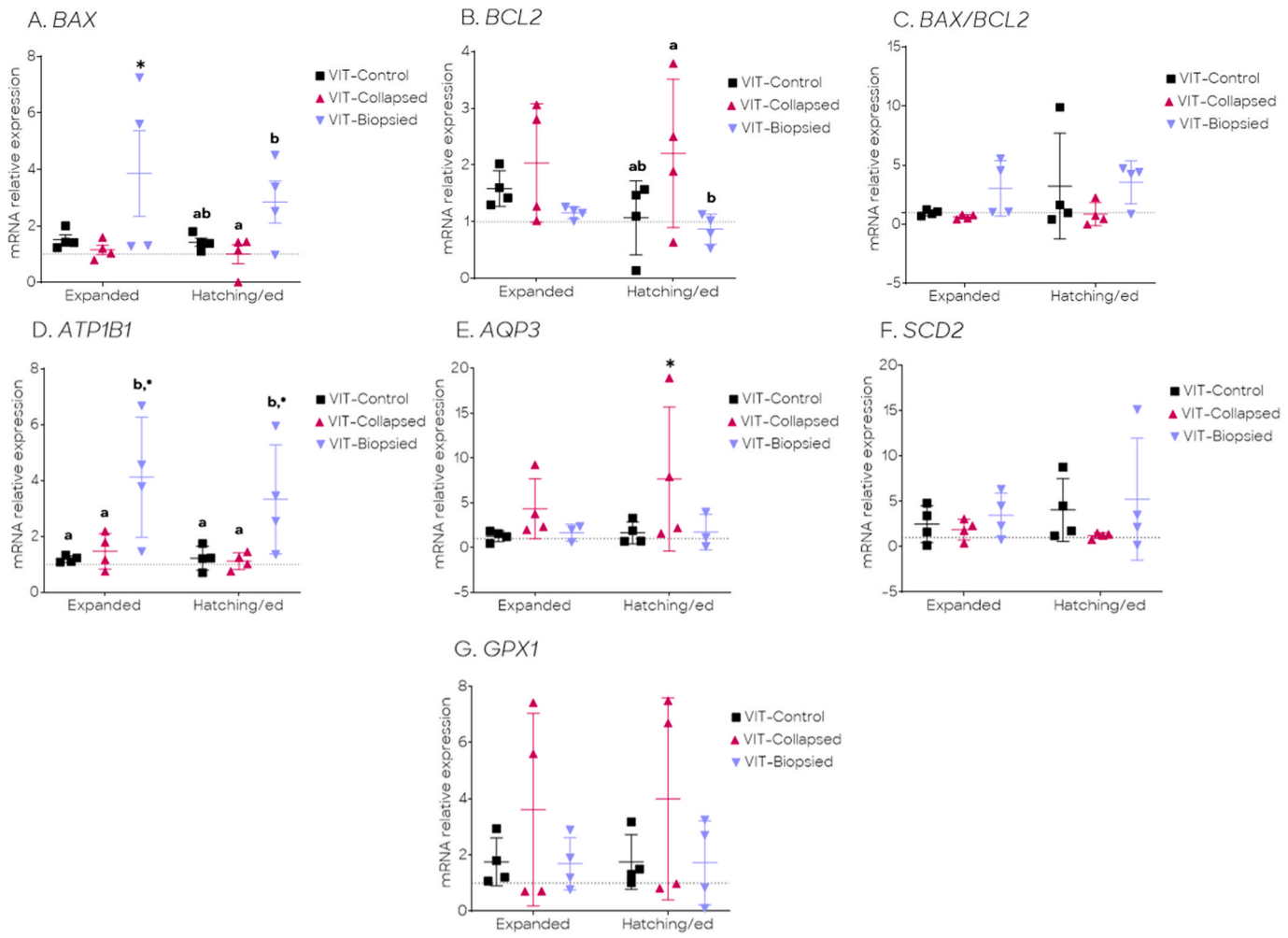

Fig. 3. Relative expression of (A) *BAX*, (B), *BCL2*, (C) *BAX/BCL2* ratio, (D) *ATP1B1*, (E) *AQP3*, (F) *SCD2*, and (G) *GPX1* genes in bovine expanded and hatching/ed blastocysts vitrified/warmed in their intact form (VIT-Control; black squares), previously collapsed (VIT-Collapsed; salmon triangles) and previously biopsied (VIT-Biopsied; black triangles). Data are shown in scatter plots where each symbol represents each individual replicate value and the solid line indicates the mean.
(*) Asterisk indicates significant differences ($P < 0.05$) between treatment groups and the Fresh Control, represented with a dotted line (mRNA relative expression = 1), according to ANOVA and Tukey's test results.
(^{a,b}) Different letters indicate significant differences ($P < 0.05$) between treatment groups according to ANOVA and Tukey's test results.
BAX: BCL2 associated X apoptosis regulator, *BCL2:* BCL2 apoptosis regulator; *ATP1B1:* ATPase Na+/K+ transporting subunit β1; *AQP3:* Aquaporin 3, *SCD2:* Stearoyl-Coenzyme A desaturase; *GPX1:* Glutathione Peroxidase 1.

is one of the most important factors affecting blastocyst quality during cryopreservation [47]. The blastocoel fluid may reduce permeability to cryoprotectants, impair vitreous state achievement, and increase the risk of crystal formation and cryodamage. Previous evidence pointed out that the blastocoel volume affects post-warming re-expansion, cell proliferation, and DNA integrity [19,48]. The current study, along with other reports in bovine species [17,18,49], demonstrated that artificial collapse can reduce the deleterious effects of blastocoel cavity in blastocyst vitrification. Different to our work, these studies discarded the aspirated blastocoel fluid and cultured vitrified/warmed embryos collectively for post-warming evaluation. In contrast, in this study, collapsed vitrified/warmed embryos were individually cultured to keep traceability with their respective BF samples. To confirm the lack of impact from individual culture, preliminary experiments conducted in our laboratory indicated that culturing a single embryo for 24 h after vitrification/warming does not affect post-warming re-expansion and hatching rates compared to embryos cultured in groups (see Supplementary Table 1).

At 24 h post-warming, re-expansion and hatching rates of collapsed bovine blastocysts were around 90.0 and 45.0 %, respectively, which were comparable to fresh intact embryos and similar to those observed by other authors [17,18,49]. Min et al. observed that collapsed bovine embryos showed higher hatching rates [17,49] and lower incidence of apoptosis [49] after vitrification than intact embryos. They also found larger offspring and less abortion rates when vitrified/warmed collapsed embryos rather than intact transferred embryos were transferred [17]. Marques and colleagues [18] also reported an increase in the post warming hatching rate of collapsed embryos compared to not collapsed embryos or those cultured with 10^{-9} M melatonin prior to vitrification. Contrarily, laser assisted hatching combined with artificial blastocoel collapse did not increase survival and hatching rates of vitrified/warmed day 6–7 buffalo expanded blastocysts [24].

Regardless of the cause of collapse (artificial: induced by puncturing, aspiration, pipetting, or osmotic shock; or natural: due to temperature changes or expansion-collapse cycles), the blastocoel cavity has the potential to gradually refill and restore the blastocyst's spherical shape [25,50]. This is evidenced by the re-expansion and hatching capacity observed in Control-Collapsed embryos. The current study found that blastocysts that were artificially collapsed before vitrification had the highest hatching percentage at 24 h post-warming when compared to other vitrified groups, highlighting the embryos' ability to recover even after the vitrification/warming process. The artificial collapse with an

ICSI pipette may disrupt the *zona pellucida*, allowing CPAs to enter and diffuse into the embryo, increasing dehydration of embryonic cells and reducing ice crystal formation. In addition, the artificial gap made in the *zona pellucida* may aid embryo hatching, especially after the zona hardening that occurs after vitrification/warming [18].

Each embryo biopsy technique has advantages and disadvantages and has a significant impact on embryo integrity, viability and chances of pregnancy after cryopreservation [9]. The microblade biopsy was chosen for this study since it is more practical, faster, and easier to perform. Some authors also claim that the larger amount of cellular material that is harvested facilitates a higher accuracy in sexing for the microblade biopsy technique [51]. Post-warming re-expansion rates obtained in the present study after vitrification/warming of biopsied blastocysts are similar to those observed in other reports [12]. Biopsied embryos, however, showed lower re-expansion and hatchability than their collapsed counterparts. The microblade biopsy produces a severe damage of the zona pellucida, as it is sometimes literally sliced away. Technically, the removal of multiple cells from the TE leads to a decline in embryo integrity due to cell loss and the rupture of TE tight junctions [52]. This may hinder the further development of the embryo, as indicated by the low re-expansion rate observed in Control-Biopsied embryos after 3 h of culture. Despite the apparent ability of these biopsied embryos to repair the damage induced by the biopsy procedure after 24 h of culture, the vitrification process notably hampers the developmental potential of biopsied embryos. Considering the importance of the zona pellucida during CPAs equilibration in cryopreservation processes, it seems that embryo survival rate decreases as the *zona pellucida* is more razed (Cenariu et al., 2012). Based on the hatching rate as a direct marker of pregnancy chance [18], one could hypothesise that collapsed cryopreserved embryos would produce higher pregnancy rates than the biopsied cryopreserved ones. Studies in mouse models found epigenetic aberrations in the brain tissue and abnormal development and function of neurons of offspring generated from blastomere-biopsied embryos [53]. Thus, BF as a source of DNA could be less detrimental to the embryo because no embryonic cells are obtained during aspiration.

Together with *in vitro* re-expansion and hatching of vitrified/warmed embryos, evaluation of the transcript levels of specific genes are the most used criterion to assess embryo cryosurvival in the absence of recipients available for transfer [15,17,18,33]. Gene expression analysis provides important information about possible changes in the molecular profile of surviving embryos, which can be indicative of further developmental potential [54]. In the attempt to determine embryonic health *in vitro,* apoptosis is one of the most important parameters [55]. Although it is naturally occurring during embryo development, apoptosis must also be tightly regulated to prevent the loss of normal cells [56] and an increased incidence could be a response to suboptimal conditions [57], such as those imposed during vitrification/warming [58]. *BAX* is a proapoptotic gene involved in the regulation of cell apoptosis whose expression was found much higher in bovine degenerated embryos [59,60]. On the other hand, *BCL2* has an antiapoptotic effect that protects cells from DNA damage [61] and its protein is significantly overexpressed than BAX protein in morphological good quality bovine blastocysts [59]. In this study, while a higher expression of the *BAX* gene was found in hatched embryos from VIT-Biopsied embryos, *BCL2* was upregulated in VIT-Collapsed hatched embryos compared to those VIT-Biopsied. Similar results were observed by Min et al. [17], who found that the expression of pro-apoptotic *BAX* decreased in artificially collapsed bovine blastocysts, whereas that of the antiapoptotic *BCL2* gene increased. These findings suggest that the artificial collapse of blastocysts could have less impact on apoptotic events and embryo quality than TE microblade biopsy.

Along with the Na^+/K^+ ATPase, aquaglyceroporin AQP3 participates in the formation and expansion of blastocyst cavity by enhancing cell permeability, which allows water movement across the TE. During cryopreservation, the blastocoel cavity suffers from an initial collapse followed by re-hydration when exposed to CPAs. These osmotic movements, which occur in intact blastocysts and involve aquaporins and ATPase, are exogenously induced in the case of artificially collapsed or biopsied embryos. When evaluating the expression of the gene encoding for the Na^+/K^+ ATPase β1-subunit (*ATP1B1*), the greatest expression was observed in biopsied vitrified/warmed embryos, whereas similar mRNA levels were found in embryos collapsed, not collapsed before vitrification and fresh controls. This fact could point out to a possible effect of microblade TE biopsy over the Na^+/K^+ ATPase activity, that would not be exerted by artificial collapse or vitrification/warming. The present findings are consistent with previous literature in mouse and cattle, where no changes in *ATP1B1* expression were found before and after vitrification/warming of intact [62] or collapsed [22] blastocysts. Similarly, whereas *AQP3* gene expression was not affected by vitrification/warming or artificial collapse, it was upregulated in hatched blastocysts surviving biopsy and vitrification/warming when compared to fresh controls. As the authors are unaware of any report on the *ATP1B1* or *AQP3* expression in biopsied embryos, it can be speculated that upregulation of *ATP1B1* and *APQ3* in biopsied blastocysts could indicate that these embryos need an extra effort to re-expand after vitrification/warming. As the correct functioning of Na^+/K^+ ATPases demand elevated production of ATP molecules, a high percentage of embryos with cryo-induced mitochondrial damage would not be able to survive vitrification [20].

5. Conclusions

The data presented in this article demonstrate that there is amplifiable DNA in the blastocoel fluid of D7 expanded bovine IVP embryos. This cfDNA is a reliable source of genetic material producing good sexing efficiency and accuracy, thus avoiding invasive procedures such as microblade TE biopsy, and reducing the associated potential risks. Given the less invasive nature of BF sampling, additional investigations are necessary to ascertain the adequacy of DNA quality and quantity within BF samples for comprehensive genomic screening of preimplantation embryos, particularly in the context of genotyping for paternity or production markers. Furthermore, the present research supports that artificial collapse offers a powerful approach to embryo cryopreservation because it improves post-warming survival and quality compared to vitrification/warming of intact and biopsied embryos. Transfers to recipient cows to assess the potential application of cryopreserved sexed embryos after blastocoel fluid aspiration are warranted.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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CRediT authorship contribution statement

Iris Martínez-Rodero: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Albert Salas-Huetos:** Validation, Supervision, Investigation, Formal analysis. **Judith Diaz-Muñoz:** Investigation. **Erika Alina Ordóñez-León:** Investigation. **Tania García-Martínez:** Investigation. **Marc Yeste:** Writing – review & editing, Validation, Supervision, Formal analysis. **Carlos Olegario Hidalgo:** Resources. **Teresa Mogas:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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List of abbreviations

cfDNA	Cell-free DNA
ANOVA	Analysis of variance
AQP3	Aquaporin 3
ATP1B1	ATPase Na+/K+ transporting subunit β1
BAX	BCL2 associated X apoptosis regulator
BCL2	BCL2 apoptosis regulator
BF	Blastocoel fluid
BRY4a	Repetitive sequence specific of Y-chromosomal DNA sequence
BSA	Bovine serum albumin
COCs	Cumulus-oocyte complexes
CPAs	Cryoprotectants
Ct	Threshold cycle
D7	Day 7
D8	Day 8
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
EG	Ethylene glycol
ES	Equilibration solution
FBS	Foetal bovine serum
Fw	Forward
g	gram
GPX1	Glutathione Peroxidase
H3F3A	H3.3 histone A
h	hour
HK	housekeeping
HM	Holding medium
Hpi	hours post-insemination
ICM	Inner cell mas
ICSI	Intracytoplasmic sperm injection
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro*-produced
M	Molar
mg	milligram
min	minute
mL:	milliliter
mm	millimetre
mM	millimolar
NaCL:	Sodium chloride
NCBI	National Centre for Biotechnology Information
ng	nanogram
°C	Celsius degrees
PBS	Phosphate-Buffered Saline
PGT	Preimplantation genetic testing
PPIA	Peptidylprolyl Isomerase A
qPCR	quantitative PCR
RNA	ribonucleic acid
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RT	retrotranscription
Rv	Reverse
sec	seconds
SAT1	spermidine/spermine N1-acetyltransferase 1
SCD2	Stearoyl-Coenzyme A desaturase
SEM	Standard error of the mean
SOF	Synthetic oviductal fluid
TCM-199	Tissue culture medium-199
TE	Trophectoderm
VS	Vitrification solution
v/v	vol/vol
WGA	Whole Genome Amplification
WS	Washing solution
µl	Microliters

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.01.042.

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