Effect of sex on cryotolerance of bovine embryos produced in vitro

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ABSTRACT

Male and female embryos are known to be different in developmental kinetics, metabolism, gene expression, and epigenetic patterns. Therefore, the objective of this study was to clarify whether the morphological criteria used to select embryos for cryopreservation lead to a deviation in the male:female ratio, and whether vitrification effects vary according to embryo sex. Initially, five sires were tested to evaluate the effect of the bull on embryo development, sex ratio, speed of development, and response to cryopreservation. Results showed that bulls affected (P<0.05) embryo production, response to cryopreservation, and sex ratio. Then, one bull was selected, and used to produce embryos in vitro to characterize the responses of male and female embryos to vitrification. Results suggested that male and female embryos have the same morphological responses to vitrification, as no differences (P>0.05) were observed between the two sexes in post-warming survival and re-expansion rates. However, their molecular responses as evaluated by gene expression (FOSL1, HSPB1, CASP3, CASP8, HSPA5, HSPA1A, G6PD, and PGK1) analysis indicated an effect of sex on vitrification; vitrified female embryos exhibited higher mRNA levels of HSPA1A, CASP3, and G6PD compared to their male counterparts. In conclusion, bulls affected embryo production, speed of development, sex ratio, and response to cryopreservation. Male and female embryos differed in their molecular responses to vitrification; and also, deviations in the male:female ratio when selecting embryos for cryopreservation were confirmed.

1. Introduction

In vitro production (IVP) of embryos is a well-established assisted reproductive technique used worldwide in several animals, particularly in the livestock industry. Nonetheless, one of the major limitations to its widespread use is the need to keep a large number of recipient cows available for receiving the embryos produced in IVP laboratories, which greatly increases the costs involved. The main reason for this is the number of embryos produced, which is, in general, much higher than that obtained in classical embryo transfer programs. Therefore, for the efficient use of IVP, cryopreservation is an indispensable tool. In addition, cryopreservation is especially important for the national and international trade of genetics, avoiding the transport of animals, which is inviable and/or illicit in most commerce abroad, and the associated health risks. Moreover, cryopreservation is essential for the establishment of germplasm banks.

However, cryopreservation of IVP embryos of several species has not provided satisfactory results yet. From 448,113 bovine IVP embryos generated worldwide in 2016, only 27% were cryopreserved [1], indicating the modest use of this technology. The most promising results for cryopreservation of IVP bovine embryos are obtained using vitrification methods [2–7], in particular the Cryotop method [8] that minimizes negative effects and improves survival rates. Although this method is being used successfully in human oocytes and embryos, efforts to improve the results in bovines are required.

In addition to the cryopreservation method, embryo quality also affects the outcome of this biotechnology. It is well known that IVP embryos have different characteristics compared to their in vivo counterparts, which include morphology [9] and molecular profiles that ultimately affect embryonic development [10,11], cryotolerance, and pregnancy rates [10–19]. Thus, the lower quality of IVP embryos compared to that of embryos produced in vivo contributes to reduced cryoresistance. The highest pregnancy rates of cryopreserved IVP bovine embryos reported in the literature used only D7 expanded blastocysts categorized as “excellent” in quality [20,21]. Therefore, the rigorous morphological selection of embryos

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appears to be a part of the cryopreservation protocol, and is one of the main factors responsible for the success of pregnancy after transfer of IVP embryos. Among the parameters used to classify IVP embryos, developmental speed is a decisive factor. A variety of studies have already shown that earlier cleavage and faster developing embryos are of better quality [22,23], affording higher pregnancy rates [24–27] and greater hatching rates after cryopreservation [5,28]. Thus, quality refers to not only the morphological appearance, but also to the speed of development.

In addition to the kinetics of development, male and female embryos differ in terms of metabolism, gene expression [19,29–31], epigenetic patterns [32], and stress responses [33,34]. Differences in how female and male embryos deal with various types of stress have been reported elsewhere. Higher levels of apoptotic cells were observed in female embryos regardless of the culture conditions; however, with a more pronounced difference in Fetal Calf Serum (FCS) medium [35]. Male embryos were more resistant to oxidative stress in FCS-supplemented medium, while the effect on blastocyst cell numbers tended to be higher in female blastocysts after stress induction [30]. Therefore, in order to evaluate how sex can influence the success of cryopreservation, it is important to identify not only if there is a gender bias when selecting embryos, but also if sex can influence the response of IVP embryos to cryopreservation.

Considering all this information together, we raised a hypothesis that morphological selection of better-quality embryos on D7 for cryopreservation may result in a preference for male embryos, causing deviations in the sex ratio.

2. Materials and methods

Unless indicated otherwise, all the reagents used were purchased from Sigma Aldrich (St. Louis, MO, USA). Cryotop devices were acquired from Vitri-Ingá (Ingáed – Maringá, PR, Brazil).

2.1. Experimental design

2.1.1. Experiment I – Effect of bull on embryo development, cryotolerances, and sex ratio

To evaluate the effect of the male on embryo sex and cryotolerance, embryos at blastocyst (BL) stage – which means only embryos on stage between early blastocyst (EBL) and expanded BL (EXBL), were vitrified, and after warming, they were evaluated for re-expansion and evolution (change in developmental stage). Five Nellore (Bos taurus indicus) bulls (B1–B5) were used for in vitro fertilization (IVF), and embryos of each bull were distributed among two groups: Control (C), n = 975 and Vitrified (V), n = 785. Cleavage on D2 and blastocyst rates on D6, 7, and 8 were observed.

To better characterize morphological responses to vitrification stress, only BL embryos (145–156 h post-insemination) were used. After warming, embryos from both treatments (C and V) were placed back into an incubator, and 4 and 24 h later, embryos were assessed for survival, re-expansion, and development rates. In all replicates, the embryos that reached expanded BL (EXBL) stage on D6 (n = 125), D7 (n = 181), and D8 (n = 140) were removed and stored in PBS and lysis buffer at −20 °C for sex identification. In this experiment, for sex analyses, genomic DNA from whole embryos was used for PCR amplification.

2.1.2. Experiment II – Effect of sex on the responses to vitrification by bovine embryos

To characterize the responses of male and female embryos to Cryotop vitrification, only one bull was used to produce the embryos. A bull (B5) showing male:female ratio closer to 50% on D7, and not presenting differences (P < 0.05) between C and V treatments in the previous experiment (experiment I) was selected. Aiming to simulate the routine used in commercial laboratories using IVP embryos for cryopreservation, only EXBL stage embryos on D7 were distributed into two groups: C (n = 129) and V (n = 165), and at 24 h post-warming, embryos were assessed for survival rates. All embryos were individually stored and used for DNA/RNA isolation simultaneously. The DNA was used for sex identification, and RNA for gene expression analysis by RT-qPCR. For gene expression analyses, only RNA from embryos that survived and were gender identified were pooled in 3 biological replicates of RNA from 25 embryos of each treatment (C male, C female, V male and V female).

2.2. In vitro Embryo production

Embryos were produced from oocytes recovered from slaughterhouse ovaries, which were transported to the laboratory in saline solution (0.9% NaCl) supplemented with amikacin (0.075 mg/mL). Follicles between 2 and 8 mm in diameter were aspirated with the aid of a syringe and a hypodermic needle (18 G). Only cumulus-oocyte complexes (COCs) presenting with homogenous cytoplasm and at least three layers of cumulus cells were used.

After aspiration and selection, oocytes were transferred in groups of 25–30 to in vitro maturation (IVM) medium. The IVM medium consisted of TCM 199 Earl’s salts (Invitrogen® – Thermo Fisher, Waltham, Massachusetts, USA), supplemented with 10% fetal bovine serum FBS (Invitrogen®), 24 IU luteinizing hormone (LH), 0.01 IU/mL follicle stimulating hormone (FSH), 0.1 mg/mL l-glutamine, 1 μM cysteamine, 1 μM sodium pyruvate and 0.075 mg/mL amikacin sulphate. Maturation was performed in 150 μL droplets covered by silicone oil for 22–24 h at 38.8 °C and 5% CO2 in air.

For IVF, frozen semen from five Nellore bulls, previously tested, was used. Motile spermatozoa were obtained using the Percoll (GE Healthcare, Piscataway, NJ, USA) gradient method in micro-tubes (Machado et al., 2009), and were added to the fertilization drop at a final concentration of 1 × 10⁶ spermatozoa/mL. Spermatozoa and oocytes were co-incubated for 14–16 h at 38.8 °C in 5% CO2 in air. Fertilization medium consisted of Tyrode’s albumin lactate pyruvate (TALP) medium [36] supplemented with 2 mM penicillin-streptomycin, 1 mM hypotaurine, 250 mM epinephrine and 10 μg/mL heparin. The day of in vitro insemination was considered as day 0.

After the co-incubation period, presumptive zygotes were washed and transferred to 200 μL drops of synthetic oviduct fluid (SOF) medium [37], supplemented with essential and non-essential amino acids, 0.34 mM sodium tricitrate, 2.77 mM myo-inositol, and 5% SFB (Invitrogen®). Embryos were evaluated on day 2 (48 h post-insemination (pi)) for cleavage, and between D6 and D8 (144–156 h pi), D7 (168 h pi) and D8 (192 h pi) for blastocyst rate determination.

2.3. Embryo vitrification and warming

Embryo vitrification was performed as previously described by Ref. [8], with minor modifications. A holding medium (HM) was used to handle embryos during vitrification and warming procedures, and was composed of HEPEs buffered TC-199 (Invitrogen®) supplemented with 20% FBS. For vitrification, the groups of embryos were first washed three times in an equilibrium solution composed of 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (Me₃SO) dissolved in HM for a minimum of 9 min. Embryos were then transferred to a vitrification solution consisting of 15% EG, 15% Me₃SO, and 0.5 M sucrose in HM, and incubated for 45–60 s. Next, embryos were placed in a Cryotop device in sets of 3–5 under a stereomicroscope. Subsequently, the Cryotop device was immediately submerged in liquid nitrogen. Warming was performed after
vitrification by immersing the end of the Cryotop for 1 min in a drop of HM supplemented with 1 M sucrose that had been pre-warmed to 37 °C. The embryos were transferred to HM supplemented with 0.5 M sucrose for 3 min and then finally transferred to original HM.

The group C embryos were maintained on the bench in HM at 37 °C while the vitrification—warming process was performed on the embryos of the V group. The vitrified embryos remained in the liquid nitrogen for at least 30 min before being warmed. Subsequently, the embryos from both C and V groups returned to SOF medium and were cultured for an additional 4 or 24 h, depending of the experiment. At the end of the culture period, embryos were evaluated for re-expansion and evolution rates. All embryos presenting no morphologically degenerating signals were considered as having survived. Embryos that changed their developmental stage during the culture period between vitrification and post-warming evaluation were considered for evolution rate calculations. The embryos that survived at 4 or 24 h (depending on the warming evaluation were considered for evolution rate calculations. All embryos presenting no morphologically degenerating signals were considered as having survived. Embryos that changed their developmental stage during the culture period between vitrification and post-warming evaluation were considered for evolution rate calculations. The embryos that survived at 4 or 24 h (depending on the experiment), were placed in RNA-later Stabilization Solution.

The group C embryos were maintained on the bench in HM at 37 °C while the vitrification—warming process was performed on the embryos of the V group. The vitrified embryos remained in the liquid nitrogen for at least 30 min before being warmed. Subsequently, the embryos from both C and V groups returned to SOF medium and were cultured for an additional 4 or 24 h, depending of the experiment. At the end of the culture period, embryos were evaluated for re-expansion and evolution rates. All embryos presenting no morphologically degenerating signals were considered as having survived. Embryos that changed their developmental stage during the culture period between vitrification and post-warming evaluation were considered for evolution rate calculations. The embryos that survived at 4 or 24 h (depending on the experiment), were placed in RNA-later Stabilization Solution (Ambion® — Thermo Fisher, Walthan, Massachusetts, USA) and stored at −80 °C until DNA or RNA extraction. Degenerated embryos were also stored for sex evaluation.

### 2.4. Genomic DNA and total RNA isolation

For each individual embryo, genomic DNA and total RNA were isolated simultaneously using the All Prep DNA/RNA Mini kit (Qiagen®, Hilden, Düsseldorf, Germany), according to the manufacturer’s instructions. The isolated genomic DNA was used for embryo sex identification. After sex identification, total RNA was pooled by sex and treatment (C and V) in groups of 25 embryos to be used for gene expression analyses.

### 2.5. Embryo sex evaluation

Sex identification was performed using a multiplex polymerase chain reaction (PCR), according to de Sousa and coworkers [38]. Briefly, the embryos were exposed for 5 min at 50 °C in a lysis solution containing 1X PCR buffer and 15 μg of proteinase K (Invitrogen®) in a final volume of 10 μL, and the enzyme was then inactivated at 95 °C for 5 min. A second step was performed by the addition of PCR mix containing 50 nM of bovine autosomal primers and 75 nM Y chromosome-specific primers, 200 μM dNTPs, 1X PCR buffer and 1U Platinum® Taq Polymerase (Invitrogen®), in a final volume of 30 μL for each sample, and placed individually in microtubes. The PCR program used consisted of heating at 94 °C for 2 min, 40 cycles of 95 °C for 30 s, 57 °C for 40 s and 72 °C for 40 s, followed by a final extension at 72 °C for 3 min. The PCR products were visualized using 1.5% agarose gels stained with ethidium bromide (10 mg/mL) under ultraviolet light. Bovine genomic DNA from a male and a female were used as positive and negative controls, respectively. For the purposes of analyzing the results, when two amplicons of 280 (autosomal) and 210 (Y chromosome) base pairs were detected, the embryo was deemed male, while with the detection of only one amplicon of 280 base pairs, the embryo was considered to be female.

### 2.6. Gene expression quantification by real time quantitative PCR (qPCR)

For gene expression analysis, the relative abundance of transcripts for eight target genes related to cellular damage and apoptosis [FOS-like antigen 1 (FOSL1), heat shock 27-kDa protein 1 (HSPB1), caspase 3 (CASP3), caspase 8 (CASP8)]; thermic stress (heat shock 70-kDa protein 5 (HSPA5)] and heat shock protein family A (Hsp70) member 1A (HSPA1A)]; glucose metabolism [glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase 1 (PGK1)] were quantified by qPCR for vitrified and fresh embryos.

The qPCR amplification was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) platform. Three biological replicates of 25 embryos for each treatment were used. The total volume of each RNA sample was used for complementary DNA synthesis using 200 U of SuperScript III reverse transcriptase (200 U/1 ml; Invitrogen®) and 0.5 μg of oligo-dT primer (0.5 μg/ml; Invitrogen®) in a final volume of 25 μL. The reactions were performed at 65 °C for 5 min, and 42 °C for 52 min, followed by enzyme inactivation at 70 °C for 15 min. qPCR analysis was performed using Fast SYBR Green Master Mix (Applied Biosystems). Reactions were optimized to provide the maximum amplification efficiency for each gene (90%—110%) on the basis of calculations using the relative standard curves in the 7500 software 2.0.3 (Applied Biosystems). Each sample was analyzed as technical duplicates, and the specificity of each PCR product was determined by melting-curve analysis and evaluation of amplicon sizes using agarose gels. Each reaction was performed in a final volume of 25 μL using complementary DNA corresponding to 0.8 embryos. The PCR cycling conditions were 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s, and then annealing at 60 °C for 30 s. Nomenclature, primer sequences and concentrations, amplicon sizes, annealing temperatures and efficiency for each primer pair are listed in Table 1. The expression levels of two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB), were submitted to the GeNorm analysis program (Vandesompele et al., 2002), which indicated that both the genes presented similar stability, with GAPDH chosen as the reference gene for data normalization [39]. The relative expression of each gene was calculated using the ΔΔCt method, with efficiency correction performed as described by Pfaffl [40].

### 2.7. Statistical analysis

The general data (the sum of all results) for comparison of blastocyst, survival, and expansion rates post-thawing, were analyzed by ANOVA-Tukey tests. Embryo rate data for evaluation of bulls were analyzed by Mann-Whitney tests. Sex frequency data were analyzed by Chi-square tests. Gene expression data were compared by Student’s t-test. All statistical analyses were performed using the Prophet Program, version 5.0, 1997, Action Stat Pro, or GraphPad Prism 6, considering P values ≤ 0.05 to be statistically significant.

### 3. Results

#### 3.1. Experiment I. Effect of bull on embryo development, cryotolerance, and sex ratios

In this experiment, five bulls were used to produce embryos, and the results of embryo development are presented in Table 2. Cleavage rates were similar (P > 0.05) for most of the bulls, except for B2, which showed a lower rate (P < 0.05) than the others. This difference was maintained during embryo development, as B2 showed the lowest (P < 0.05) blastocyst rate on D6, D7, and D8. No differences (P > 0.05) in the kinetics of development were observed among bulls.

Cryotolerance, as evaluated by morphological characteristics, showed that no differences among bulls were observed when C or V groups were compared (P > 0.05). However, when each bull was analyzed individually, embryos derived from B2 and B5 bulls showed the best survival rates after vitrification as shown by
decrease in the proportion of degenerates and increase in the proportion of EBLs after 24 h culture (Table 3), and these two had no difference between them (P > 0.05).

To evaluate the effect of the bull on male:female ratios, embryos that reached the EBL stage on D6, D7, and D8 were removed from culture and sexed. When the individual effect of the bull was disregarded, no differences were found in terms of the percentage of male and female embryos on EBL (Fig. 1). However, a higher (P < 0.05) percentage of male than female embryos was evident on EBL at D7 and D8. Considering each bull individually, only B2 and B4 showed different proportions of development than expected of male:female embryos on D7.

3.2. Experiment II. Effect of sex on the responses to vitrification of bovine embryos

In this experiment, only one bull (B5), selected from the results of experiment I, was used to evaluate the effect of sex on the morphological and molecular responses to vitrification. Embryo production data involving the selected bull are depicted in Table 4. Cleavage and blastocyst rates by D6 were similar to those obtained in experiment I. On D7, approximately 62% of embryos were at the early blastocyst stage, of which 89.8% were considered class I embryos and qualified for vitrification.

Considering that no differences were found between 4 h and 24 h post-warming regarding to morphological characteristics in the first experiment, embryos for gene expression in this trial were collected at 24 h post-warming. At 24 h post-warming, some embryos degenerated, and the percentage of degenerated embryos was similar (P > 0.05) between C and V treatments (Table 4). Additionally, a majority of the embryos had hatched BL (HBL) in both treatments; however, group C had more embryos at the HBL stage than group V (P < 0.05). When the sex of the embryos was considered (Fig. 2), the kinetics of development and male:female ratios were similar between the groups V and C.

To evaluate the molecular responses to vitrification according to sex, mRNA levels of eight genes were quantified (Fig. 3). In group V, female and male embryos had different transcript levels for HS2B1A (P = 0.0043), CASP3 (P = 0.0037), and G6PD (P = 0.0071) genes, whereas in group C, a difference in transcript levels was observed only for the G6PD gene (P = 0.0526).

4. Discussion

Cryotolerance is clearly related to embryo quality, and this quality can be evaluated by the speed of development. Given that male and female embryos differ in their developmental kinetics, we raised the hypothesis that by selecting the best quality embryos for cryopreservation, we may be inducing male-biased selection. To test this hypothesis, we evaluated if sex affects IVP embryo kinetics and their responses to vitrification stress.

Considering that bulls can also influence embryo sex ratios, initially we evaluated the effects of the bulls on the developmental kinetics and cryotolerance of embryos. To achieve this, we used five

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**Table 1**

Information regarding specific primers used for PCR amplification of gene fragments for qPCR analysis.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers sequences</th>
<th>Amplicon size (bp)</th>
<th>Primer Concentration (nM)</th>
<th>Primer Efficiency (%)</th>
<th>GeneBank Access number/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: GCC GTG AAC CAC GAG TAT AA</td>
<td>118</td>
<td>300</td>
<td>94.57</td>
<td>NM_001034034.2</td>
</tr>
<tr>
<td></td>
<td>R: CCC TCC ACG ATG CCA AAG T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>F: GCC ACC CAC CAC AAT GAT CAA</td>
<td>126</td>
<td>300</td>
<td>100.15</td>
<td>XM_010845770.1</td>
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<tr>
<td></td>
<td>R: ATC GTA CTC GTC CTT GAT CCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASP3</td>
<td>F: GCC GAG TCC TGT AGC GGT CA</td>
<td>185</td>
<td>250</td>
<td>105.3</td>
<td>NM_001077840.1</td>
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<tr>
<td></td>
<td>R: AAA TGT GAC CCG CTT TGT T</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASP8</td>
<td>F: CAG AAC AGA TGG AAG CCT AT</td>
<td>209</td>
<td>250</td>
<td>101.8</td>
<td>NM_0010450970.2</td>
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<td>R: GGT TAG GAT CCT GAG AAT GT</td>
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<td>HS2B1A</td>
<td>F:CAA GAT CAC CAT CAC CAA GG</td>
<td>219</td>
<td>300</td>
<td>90.98</td>
<td>NM_170450.1</td>
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<td>R:AAA TCA CCT CCT GGC ACT TG</td>
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<tr>
<td>HS2B5</td>
<td>F: CCT GGC CAA TAA TGG AGA TAC</td>
<td>119</td>
<td>300</td>
<td>101.27</td>
<td>NM_001075148.1</td>
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<td>R: CTC TGT TGT CCT TCC GAA CAT</td>
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<tr>
<td>HS2B51</td>
<td>F: CTG GAC GTC AAC CAC TTC</td>
<td>180</td>
<td>300</td>
<td>105.31</td>
<td>NM_001025569.1</td>
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<tr>
<td></td>
<td>R: GCA CAG ACA GCA GGA GAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOS1</td>
<td>F: GCT TCC TAG TAG ACC CAA AG</td>
<td>200</td>
<td>300</td>
<td>97.70</td>
<td>NM_001205985.1</td>
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<tr>
<td></td>
<td>R: GAA GAT GTC ATG AAT ACC ATA G</td>
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<td></td>
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<tr>
<td>G6PD</td>
<td>F: GGT CCT CAA CCC CCA GGA GTC</td>
<td>183</td>
<td>300</td>
<td>97.90</td>
<td>NM_001244135.2</td>
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<tr>
<td></td>
<td>R: ATG TGC CAA CCA ACT GGA GTG</td>
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<tr>
<td>PGK1</td>
<td>F: TGC AGC TGA ATT GCC AAG ATG TCG</td>
<td>161</td>
<td>300</td>
<td>102.20</td>
<td>NM_001034299.1</td>
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<td>R: TGC ATG CTT GCA ACA GCA GCC TGG</td>
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<td></td>
<td></td>
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</tbody>
</table>

F: primer forward; R: primer reverse; bp: base pair; nM: nano molar.
different bulls and compared development, sex, and post-thawing survival rate of the embryos. Our results showed differences in total blastocyst production among the bulls. These data corroborate the work of Machado et al. (2009) [41] and others (Alomar et al. (2008); Palma et al. (2004); Ward et al. (2001), respectively: [34,42,43]) who also observed an effect of the bull on cleavage and blastocyst rates of development on D7 and D8. In fact, various authors have reported individual variation among bulls in terms of their ability to produce embryos in vitro [34,42-46].

Concerning developmental kinetics, we found no differences among bulls. It is difficult to compare our data with that of others, not only due to the scarcity of studies evaluating these parameters, but also because of differences in methods and systems adopted by the various authors. Nevertheless, Alomar et al. [34], who evaluated embryonic development by time-lapse imaging, using 4 bulls, reported no differences among bulls with respect to embryo developmental kinetics. Similar results were also reported when sexed sperm [47] or media enriched with glucose and fructose to improve embryo quality [48] were used.

Regarding bull effects on IVP embryo cryotolerance, we found it interesting that the same two males (B2 and B5), whose V embryos showed better survival after the vitrification process, also exhibited no differences in survival rates between C and V embryos. These results may indicate a paternal component in embryo cryotolerance, suggesting that information of the bull has to be considered when IVP embryos are to be cryopreserved. To our knowledge, no studies in cattle have reported a bull effect on embryo cryotolerance, whether of in vivo or in vitro origin. We are not sure how the parental male can affect embryo cryopreservation resistance, but it is possible that some type of sperm DNA damage, or perhaps epigenetic alteration may be involved [49].

To evaluate the effect of bull on male:female ratios, embryos that reached the EXBL stage of development on D6, D7 and D8 were sexed. From the five selected bulls, two presented higher numbers of males compared to female embryos on D7 and D8. Only one bull exhibited no differences in the male:female ratios. When all data were analyzed together, it was observed that on D7, IVP embryos were predominantly male, and gender bias became more pronounced by D8. Previous studies have also shown that in vitro systems favor male embryos [27,41,50-53], and such differences

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**Table 3**

Effect of bulls (B) on the survival and development at 4 and 24 h post-warming, of in vitro produced embryos, vitrified at blastocyst stage on day 6 of culture.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Treatment</th>
<th>Total N (BL)</th>
<th>Survival Rate</th>
<th>Post-warming Development</th>
<th>4 h</th>
<th>24 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N %</td>
<td>N %</td>
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<td>N %</td>
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<td></td>
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<td>N %</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>N %</td>
<td>N %</td>
</tr>
</tbody>
</table>

Different letters in same column represents difference: a, b Comparing between treatments: Control and Vitrified/Bull; A, B Comparing among bulls/Treatment (Control or Vitrified), by ANOVA, Tukey test (P < 0.05). Data are from 26 experimental replicates.

N: number of structures; BL: Blastocyst stage embryo; EXBL: Expanded blastocyst stage embryo.

Percentages of survival rate are based on BL total number; percentages of post-warming development are based on total number of embryos used in respectively observation period.

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**Fig. 1.** Effect of bulls (B) on sex of in vitro produced bovine embryos at expanded blastocyst stage on days 6 (D6), 7 (D7), and 8 (D8) of development. * in columns represents differences between male and female embryos: comparison per day of embryo development and per bull, by Chi-square test (P < 0.05).
become more evident as embryos develop up to D14 and D15 [19,51]. However, our findings are not consistent with those of Larson and coworkers [54] who reported that more female embryos reached morula and expanded blastocyst stage on D6. Therefore, it seems that there is an individual variation among sires in their ability to produce more male or female embryos. It is important to point out that the culture system used (bovine calf serum and high oxygen tension) may also induce a sex deviation in the IVP embryos [51], which can be responsible by the differences found among studies.

Considering that several studies have shown that embryos developing faster in vitro are of better quality and more capable of dealing with stress conditions [5,55–58], and that female embryos are slower in development than male ones [59–61], we hypothesized that fewer female embryos would be selected to be cryopreserved. Therefore, to assess whether we are inducing a sex bias when selecting embryos for vitrification, and if sex affects vitrification responses, we used only one bull to produce embryos. B5 was selected for this purpose as it showed no differences in terms of male:female embryo ratios on D7, and also displayed no differences in embryonic survival and development after vitrification. To mimic the procedures used in commercial laboratories for cryopreserved IVP embryos, only EXBL grade I of D7 were vitrified [7,21]. Survival rates did not differ between C and V embryos; however, the evolution and hatching rates were lower for the V group than for C. When the effect of sex on the vitrification response was evaluated, our results showed that cryptotolerance was similar between genders for all morphological features. However, in contrast to our results, the literature reports that male and female embryos have different behaviors when exposed to stressful conditions, such as during embryo biopsy, and female embryos need more time for re-expansion [60]. In addition, Nedambale et al. [62] showed that male embryos, besides developing faster than females, responded better to vitrification stress, with higher survival and hatching rates post-thawing. Although few experiments were conducted with vitrified and sexed embryos in cattle [63], we were expecting to observe differences between genders in response to vitrification.

Similar to post-thawing development, gene expression has also been widely used to evaluate embryo quality [13,29,64]. Therefore, we also used this assessment tool to verify gender differences in response to cryopreservation stress. Of the eight genes investigated, three (CASP3, HSPA1A, and G6PD) were expressed at different levels among the groups. Female vitrified embryos showed greater abundance of CASP3, HSPA1A, and G6PD gene transcripts than male vitrified embryos. However, no evident differences were observed between genders before and after cryopreservation.

The apoptotic cascade involves a variety of enzymes which induce programmed cell death by different pathways. These include initiator caspases (CASP2, CASP8, and CASP9) and effectors (CASP3, CASP6, and CASP7). Although we evaluated one from each group, CASP8 and CASP3, CASP3 was expressed at different levels in female and male vitrified embryos. Apoptosis may allow embryos to survive stress by removal of damaged cells, and it may be that, as a consequence, embryo developmental competence depends on apoptosis extension. Apoptotic process during preimplantation development allows the elimination of cells with abnormal or detrimental potential and has both beneficial and detrimental effects, depending of the timing and the level of the process. In fact abnormal embryo development has also been associated with

### Table 4

**Survival rate and evolution of expanded blastocysts (EXBL) vitrified by the Cryotop method at day 7 and evaluated 24 h post-warming.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total EXBL</th>
<th>Survival Rate</th>
<th>Evolution on 24 h post-warming</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>EXBL (%)</td>
<td>HBL (%)</td>
</tr>
<tr>
<td>Control</td>
<td>130</td>
<td>130 (100%)</td>
<td>20 (15.4%)</td>
</tr>
<tr>
<td>Vitrified</td>
<td>231</td>
<td>224 (96.9%)</td>
<td>108 (46.8%)</td>
</tr>
</tbody>
</table>

a, b Different letters on same column represents difference between treatments (Control and Vitrified), by t-test ($P < 0.05$). Data are from 6 experimental replicates.

N: number of structures; EXBL: expanded blastocyst stage; HBL: hatched blastocyst stage. Percentages are based on total EXBL number.

![Fig. 2](image-url)  

**Fig. 2.** Sex frequency of vitrified bovine embryos on day 7 of development and evaluated 24 h post-warming. Comparison between male and female embryos by developmental stage, by Chi-square test ($P < 0.05$). EXBL: expanded blastocyst stage; HBL: hatched blastocyst stage; Dg: degenerated embryos.
altered CASP-3 levels [65]. The fact that cryopreserved female embryos had higher transcript levels of CASP3 than male ones indicates that differences between genders are exacerbated under stressful conditions.

Accumulating evidence shows that HSPA1A, an inducible heat shock protein that essentially functions as a molecular chaperone to maintain cellular homeostasis, is an important cellular stress marker [66–68]. In addition, HSPA1A has been shown to interact with and modulate stress-induced apoptotic pathway molecules [68]. The induction of HSP expression starts a few minutes after thermal stress episodes, and its peak expression may occur up to several hours after the exposure [69]. Mori and coworkers [66] suggest that the freezing and thawing process increases blastocyst damage, resulting in increased expression of HSPA1A.

Therefore, the higher levels of CASP3 and HSPA1A transcripts in female vitrified embryos, compared to male ones, may suggest that females are more susceptible to such stress than males. In addition, the presence of higher transcript levels of these genes emphasizes that sex-related differences are likely to be greater under suboptimal conditions [70].

Fig. 3. Relative abundance of mRNA encoding FOSL1, HSPB1, HSPA5, HSPA1A, CASP3, CASP8, G6PD, and PGK1 determined by qPCR in non-vitrified and vitrified bovine expanded blastocysts. Mean ± standard deviation (SD) of the mean of three biological replicates. The data (mean ± SD) were normalized using the ΔΔCT method [40], with GAPDH as an endogenous control. * indicates statistically significant differences between treatments obtained by Student’s t-tests (P ≤ 0.05).
overexpressed in female bovine embryos, compared to male embryos [51,64,71]. This was the gene uniquely and significantly different in fresh female and male embryos, and this variance persisted after the cryopreservation process. Female embryos were not affected by vitrification stress, demonstrating their good quality due the activation of G6PD being indicative of response to oxidative stress and, in bovine blastocysts, may constitutively depend on embryonic gender [71–73]. Under stress or oxidative stress conditions, it has been reported that female embryos are favored by the different gene expression of G6PD [71,74].

Results found in our study suggest that selection of the bull affects embryo production, kinetics of development, sex, and responses to cryopreservation. Furthermore, bulls that produce more cryoresistant embryos are not always those that produce higher IVP blastocyst rates. Hence, our concern of inducing a deviation in gender ratios when selecting embryos for cryopreservation is not unfounded, with the bias being more related to the choice of bull for IVP systems. Despite all the differences between sexes reported in the literature, our data suggested that male and female embryos had the same vitrification responses with respect to morphological post-thawing evaluation; however, differed in their molecular responses. Such differences may appear later in development and manifest effects on pregnancy rates or gestation maintenance. The time point when differences emerge between male and female embryos, which affect cryoresistance, should also be evaluated.

Conflicts of interest
The authors declare no conflicts of interest.

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