Zygote

cambridge.org/zyg

Research Article

Cite this article: de Mattos K *et al*. (2022) β-Mercaptoethanol in culture medium improves cryotolerance of *in vitro*-produced bovine embryos. *Zygote*. **30**: 830–840. doi: 10.1017/S0967199422000338

Received: 28 December 2021
Revised: 12 June 2022
Accepted: 13 June 2022
First published online: 23 September 2022

Keywords:
Antioxidant; Cattle; Cryopreservation; Cryosurvival; IVP embryo

Author for correspondence:
Eduardo de Souza Ribeiro. Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada N1G 2W1.
E-mail: eribeiro@uoguelph.ca

*Present address: Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada N1G 2W1

© The Author(s), 2022. Published by Cambridge University Press.

β-Mercaptoethanol in culture medium improves cryotolerance of *in vitro*-produced bovine embryos

Karine de Mattos[1], Camilo Andrés Pena-Bello[1], Karine Campagnolo[1], Gabriella Borba de Oliveira[1], Elvis Ticiani[1], César Augusto Pinzón-Osorio[1], Ana Laura da Silva Feijó[1], Higor da Silva Ferreira[1], José Luiz Rodrigues[1], Marcelo Bertolini[1], Alceu Mezzallira[2] and Eduardo de Souza Ribeiro[2,*]

[1]School of Veterinary Medicine, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil and [2]Center of Agroveterinarian Sciences, Santa Catarina State University, Lages, SC, Brazil

Summary

The objective of this study was to investigate the effects of adding β-mercaptoethanol (βME) to culture medium of bovine *in vitro*-produced (IVP) embryos prior to or after vitrification on embryo development and cryotolerance. In Experiment I, Day-7 IVP blastocysts were vitrified and, after warming, cultured in medium containing 0, 50 or 100 µM βME for 72 h. Embryos cultured in 100 µM βME attained higher hatching rates (66.7%) than those culture in 0 (47.7%) and 50 (52.4%) µM βME. In Experiment II, IVP embryos were *in vitro*-cultured (IVC) to the blastocyst stage in 0 (control) or 100 µM βME, followed by vitrification. After warming, embryos were cultured for 72 h (post-warming culture, PWC) in 0 (control) or 100 µM βME, in a 2 × 2 factorial design: (i) CTRL–CTRL, control IVC and control PWC; (ii) CTRL–βME, control IVC and βME-supplemented PWC; (iii) βME–CTRL, βME-supplemented IVC and control PWC; or (iv) βME–βME, βME-supplemented IVC and βME-supplemented PWC. βME during IVC reduced embryo development (28.0% vs. 43.8%) but, following vitrification, higher re-expansion rates were seen in βME–CTRL (84.0%) and βME–βME (87.5%) than in CTRL–CTRL (71.0%) and CTRL–βME (73.1%). Hatching rates were higher in CTRL–βME (58.1%) and βME–βME (63.8%) than in CTRL–CTRL (36.6%) and βME–CTRL (42.0%). Total cell number in hatched blastocysts was higher in βME–βME (181.2 ± 7.4 cells) than CTRL–CTRL (139.0 ± 9.9 cells). Adding βME to the IVC medium reduced development but increased cryotolerance, whereas adding βME to the PWC medium improved embryo survival, hatching rates, and total cell numbers.

Introduction

The industry of bovine *in vitro* produced (IVP) embryos has grown substantially worldwide in the past decades, with a little over a million IVP embryos produced in 2019, which represented nearly 70% of the total bovine embryos produced worldwide (Viana, 2020). Out of IVP embryos transferred to recipients, ~56% were transferred fresh and 44% were transferred following cryopreservation procedures (Viana, 2020). Despite the gradual increase in the number of cryopreserved IVP embryos transferred to recipients, IVP embryos are still considered less cryotolerant than their *in vivo*-derived counterparts (Abe *et al*., 2002; Seidel, 2006; Sudano *et al*., 2011; Gómez *et al*., 2020). Therefore, the development of reliable methods to improve cryosurvival of IVP embryos is necessary to optimize the efficiency of IVP procedures and to enhance the feasibility of using IVP embryos in breeding programmes of dairy and beef herds (Ribeiro *et al*., 2012; Leme *et al*., 2020).

The lower cryotolerance of bovine IVP embryos seems to be related to suboptimal *in vitro* culture conditions (Lonergan *et al*., 2003). The phenomenon is associated with increased apoptosis (Bain *et al*., 2011) and DNA fragmentation in IVP embryos compared with *in vivo*-produced embryos (Velez-Pardo *et al*., 2007). In addition, the elevated intracytoplasmic lipid content of IVP embryos appears to increase the sensitivity to oxidative stress and to compromise embryo quality and the response to freezing (Guérin *et al*., 2001; Seidel, 2006). Oxidative stress in embryo culture is characterized by an imbalance between oxidants and antioxidants induced by an increase in oxidative agents or a decrease in antioxidants (Rocha-Frigoni *et al*., 2014). Excessive amounts of free radicals can promote changes in proteins and lipids, resulting in DNA damage that further compromises the survival of IVP embryos to culture conditions and cryopreservation (Ray *et al*., 2012; Jamil *et al*., 2020; Lin and Wang, 2020). Oxidative modifications of cellular components represent the main stress inducer of embryos in culture and are especially important after cryopreservation because of the resulting cryo-injuries (Deleuze and

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

Goudet, 2010). Nevertheless, it is noteworthy that free radicals are also important messengers in cell signalling (Ray *et al.*, 2012). In fact, many studies have shown a key role of physiological concentration of free radicals in plasma membrane ion transport, pH changes, redox potential, oocyte development and fertilization competence, and regulation of mitotic divisions during early embryonic development, factors that ultimately determine the success in embryo development (Jamil *et al.*, 2020; Lin and Wang, 2020). Therefore, the success of IVP production and cryopreservation depends greatly on the redox homeostasis of embryos, which work in an interdependent and dynamic balance between free radicals and antioxidant compounds (Lin and Wang, 2020).

Antioxidants have a critical role in preventing excessive formation of free radicals and consequent oxidative damage (Sies, 1997). Antioxidant precursors, especially of low molecular weight, such as thiol compounds, have been used in IVP, showing favourable effects on embryo development (Mori *et al.*, 2006; de Castro e Paula and Hansen, 2008). For instance, the antioxidant β-mercaptoethanol (βME), a precursor of glutathione (GSH), has a wide range of biological actions in embryo development (Rodríguez-González *et al.*, 2003; Mori *et al.*, 2006). β-Mercaptoethanol has been shown to reduce 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH)-initiated peroxyl radicals, to inhibit the oxidation of free sulfhydryl residues, to chelate metal ions and divalent cations, and to remove hydroxyl radicals when in solution (Cornell and Crivano, 1972; Rashidipour *et al.*, 2020). Supplementation of βME in different IVP stages improved developmental competence of IVP embryos through the maintenance of oxidative homeostasis and the protection of blastomeres against the effects of oxidative lesions, increasing blastocyst rates (Takahashi *et al.*, 1993; Caamaño *et al.*, 1998; Geshi *et al.*, 1999; de Matos and Furnus, 2000; Feugang *et al.*, 2004). In addition, βME added to the culture medium after vitrification resulted in beneficial effects on bovine IVP embryos, increasing blastocyst survival, hatching rates, and total cell numbers (Nedambale *et al.*, 2006). In vitrified buffalo IVP blastocysts, βME modulated the expression of genes associated with embryo quality and antagonistic mechanisms to apoptosis (Moussa *et al.*, 2019). Nevertheless, the effects of antioxidant precursors, such as βME, on the process of cryopreservation of IVP embryos still need more attention (Nedambale *et al.*, 2006; Hosseini *et al.*, 2009; Rocha-Frigoni *et al.*, 2014). Timing of supplementation and inclusion levels according to predicted needs of the IVP system are important factors to be further investigated. Therefore, the objective of this study was to evaluate the effects of timing and concentration of βME supplementation on *in vitro* embryo culture medium on the quality, viability, and cryotolerance of bovine IVP embryos.

Materials and methods

Chemicals and experimental conditions

All reagents were from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless stated otherwise. All procedures were performed in a laboratory at room temperature.

In vitro *embryo production*

In vitro production of bovine embryos was performed according to our established standard procedures (Ribeiro *et al.*, 2009) with a few modifications. Bovine ovaries obtained at a local slaughterhouse were transported to the laboratory at 30°C in phosphate-buffered saline (PBS) solution supplemented with penicillin (100 IU/ml) and streptomycin (0.05 mg/ml) between 2 and 4 h after collection. Ovaries were washed in PBS, and cumulus–oocyte complexes (COCs) were aspirated from 3–8 mm diameter follicles with an 18G needle attached to a 10-ml syringe and pooled into 50-ml conical tubes. Only grades I and II COCs (Stojkovic *et al.*, 2001) were selected under a stereomicroscope (×15 magnification) for *in vitro* maturation (IVM). Selected COCs were transferred in groups of 40–50 structures to four-well dishes (Nunclon™, Roskilde, Denmark; cat. no. 176740) containing 400 µl of *in vitro* maturation (IVM) medium, composed of TCM 199 with Earle's salts, supplemented with 26.2 mM $NaHCO_3$, 25 mM HEPES, 0.2 mM sodium pyruvate, 5 µg/ml FSH/ml (Follitropin, Bioniche, Canada), 0.5 µg/ml LH (Lutropin, Bioniche, Canada) and 10% of inactivated estrous mare serum (EMS), under mineral oil, maintained at 39°C, 5% CO_2 in air and saturated humidity for 24 h.

Frozen semen from a previously tested *Bos taurus* bull was used for bovine IVF procedures. Sperm cells were selected by the swim-up technique in Sperm-TALP medium supplemented with 6 mg/ml bovine serum albumin (BSA), at 39°C, in a water bath. After 60 min of ascending migration, the supernatant was collected and centrifuged for 5 min at 700 *g*, with the pellet collected and diluted in Sperm-TALP medium to obtain an inseminating dose of 1×10^6 to 1.5×10^6 spermatozoa/ml matured COCs and sperm cells were co-incubated for 18–22 h (Day 0) at 38.5°C, in 5% CO_2, and saturated humidity. Fertilization medium consisted of Fert-TALP medium supplemented with 30 µg/ml heparin, 30 µg/ml penicillamine, 15 µM hypotaurine, and 1 µM epinephrine, in four-well plates with the same number of IVM structures. After fertilization (Day 1), presumptive zygotes were gently denuded by repeated pipetting in TCM-HEPES medium.

Immediately after cell removal, presumptive zygotes were washed and cultured in four-well plates containing 40–50 structures per well in 400-µl drops of SOFaaci medium (Holm *et al.*, 1999) supplemented with 6 mg/ml of BSA covered by mineral oil. In the first 24 h, embryos were cultured in 5% CO_2 in air. Cleavage rate was evaluated on Day 2 when plates were placed into an impermeable bag (Bag-system; Vajta *et al.*, 1997), under a gas mixture containing 90% N_2, 5% O_2 and 5% CO_2 in an incubator at 38.5°C and saturated humidity, for 7 days. On Day 7 of development, blastocyst rates and embryo kinetics were evaluated by morphology and according to Stringfellow and Givens (2010).

Vitrification and warming procedures

Embryo vitrification was performed as previously described by Werlich *et al.* (2006) based on open pulled straw technology. Heated and stretched glass capillaries were used to make micropipettes (glass micropipettes, or GMP). For vitrification, groups of three blastocysts of excellent or good quality (Grade 1) on Day 7 of development were first washed for 1 min with an equilibrium solution (extracellular solution) composed of 10% ethylene glycol (EG) and 10% propylene glycol (PRO) in PBS + 10% EMS, followed by a 20 s exposure to a vitrification solution (extracellular solution) composed of 20% EG, and 20% PRO in PBS + 10% EMS, at room temperature (25ºC). Subsequently, embryos were placed in a small droplet (< 2 µl), pulled inside the GMP by capillarity, and immediately submerged into liquid nitrogen.

Warming was performed 30–90 min after vitrification by exposing the GMPs to air for 3 s, and then immerging into a tube containing 1.2 ml warming solution (PBS + 10% FBS) with 0.3 M sucrose for 5 min at 39ºC. The GMP content was transferred to a

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

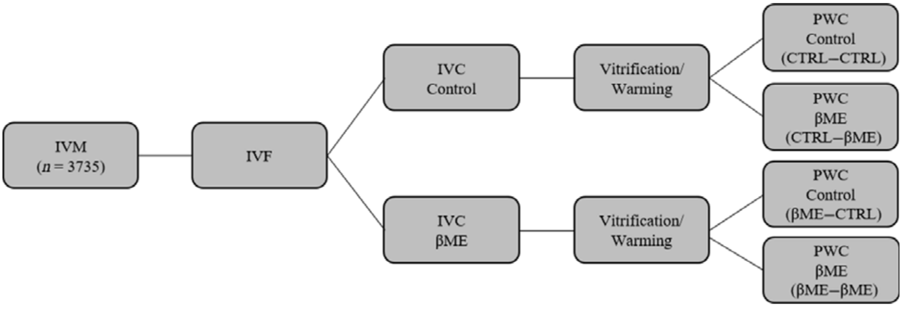


Figure 1. Treatment design of Experiment II. In total, 3735 COCs were matured and fertilized *in vitro* and then randomly allocated to be cultured in control medium (Control IVC) or medium containing 100 µM βME (βME IVC) from Day 1 to Day 7 of embryo development. Resulting Day-7 blastocysts were vitrified and, after warming, randomly allocated to post-warming culture (PWC) in control medium (control PWC) or medium containing 100 µM βME (βME PWC) for 72 h, forming four experimental groups in a 2 × 2 factorial design. CTRL–CTRL: control IVC and control PWC; CTRL–βME: control IVC and βME-supplemented PWC; βME–CTRL: βME-supplemented IVC and control PWC; βME–βME: βME-supplemented IVC and βME-supplemented PWC.

drop containing the warming solution with 0.15 M sucrose. After 5 min, embryos were segregated homogeneously in groups, according to the experiments below, and placed in *in vitro* culture in SOFaaci + 5% EMS at 38.5°C, 5% CO_2 and saturated humidity for 72 h to evaluate the *in vitro* re-expansion and hatching rates. Blastocysts reaching the hatching stage (Be) were considered viable.

Estimation of total cell number in IVP blastocysts

Estimation of the total cell numbers in embryos was performed according to Ribeiro *et al.* (2009). Hatched embryos from each treatment group were segregated by hatching day and by morphological classification, being fixed in 99% ethanol at 4°C for at least 12 h. For the estimation of cell number, fixed embryos were exposed to a solution of 15 µg/ml bisbenzimide (Hoechst 33342) in 99% ethanol for 10 min protected from direct light. Embryos were then placed in a minimum volume in a 10-µl glycerol drop on a slide, which was covered with a coverslip. Finally, embryos were examined under UV light (450–490 nm excitation, 500–540 nm emission) at ×400 magnification in an inverted fluorescence microscope (Zeiss AxioVert 135®, Oberkochen, Germany).

Experiment I: Effect of β-mercaptoethanol (βME) added to the post-warming in vitro *culture (PWC) medium on blastocyst survival after vitrification*

The effects of adding 50 or 100 µM βME to the PWC on the survival and hatching rates after 72 h were evaluated. Day-7 grade-1 vitrified blastocysts ($n = 191$) were segregated randomly to three groups, as follows: (i) 0-βME group, control group without βME supplementation in the PWC medium; (ii) 50-βME group, PWC medium supplemented with 50 µM βME; and (iii) 100-βME group, PWC medium supplemented with 100 µM βME. Embryos were *in vitro* cultured for up to 72 h, at 38.5°C, 5% CO_2, and saturated humidity, with evaluation of re-expansion and hatching rates every 24 h. β-Mercaptoethanol was added to fresh culture medium on the day of blastocyst warming, and its content was not replenished during the 72 h culture to prevent disturbance in the culture conditions and to make the proposed intervention more practical for consideration in commercial laboratories.

Experiment II: Effect of βME supplementation prior to and after vitrification on embryo survival and in vitro *development*

The effects of adding 100 µM βME to the medium prior to and/or after vitrification on the cryotolerance and viability of vitrified IVP bovine blastocysts were evaluated (Figure 1). *In vitro* culture medium was either supplemented or not with 100 µM βME from Day 1 to Day 7 of embryo development. Resulting Day-7 blastocysts were vitrified and, after warming, *in vitro* cultured in medium either supplemented or not with 100 µM βME for up to 72 h. Thus, four experimental groups were formed in a 2 × 2 factorial, as follows:

- CTRL–CTRL: control IVC and control PWC
- CTRL–βME: control IVC and βME-supplemented PWC
- βME–CTRL: βME-supplemented IVC and control PWC
- βME–βME: βME-supplemented IVC and βME-supplemented PWC.

The main effect of βME supplementation in IVC (βME–IVC) was evaluated as (CTRL–CTRL + CTRL–βME) vs. (βME–CTRL + βME–βME). The main effect of βME supplementation in PWC (βME–PWC) was evaluated as (CTRL–CTRL + βME–CTRL) vs. (CTRL–βME + βME–βME).

Cleavage rates were evaluated on Day 2, and blastocyst rates and embryo kinetics on Day 7. Re-expansion rates were evaluated 12 h after embryo warming, and hatching rates were evaluated at 24, 48, and 72 h of PWC. In addition, the morphological quality and total cell numbers were evaluated in hatched blastocysts after 72 h of PWC. Similar to Experiment I, β-mercaptoethanol was added to fresh culture medium on the day that embryos were put into culture, and its content was not replenished during the culture periods to prevent disturbance in the culture conditions and to make the proposed interventions more practical for consideration in commercial laboratories.

Statistical analyses

Data regarding cleavage and blastocyst rates, embryo morphological quality after IVP, and re-expansion and hatching rates after 12 h and 72 h of PWC, respectively, were compared between groups within each experiment by chi-squared (χ^2) test, for $P < 0.05$. Total cell numbers in hatched embryos between groups were subjected to analysis of variance, with pairwise comparisons using Tukey's test ($P < 0.05$). Statistical analyses were performed using Minitab® (State College, PA, USA).

Results

Experiment I: Effect of β-mercaptoethanol (βME) supplementation to the in vitro *culture medium after vitrification on subsequent embryo survival*

According to Table 1, the supplementation with 100 µM βME in PWC improved hatching rates of vitrified Day-7 blastocysts when compared with the control (0-βME) group (66.7% vs. 47.7%, $P < 0.05$), and both groups did not differ from the 50-βME group

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

Table 1. Bovine IVP blastocyst hatching rates following vitrification, after 72 h of post-warming *in vitro* culture (PWC) in medium supplemented with 0 (control, 0-βME), 50 μM (50-βME) or 100 μM βME (100-βME)

	Embryos in PWC	Hatching rate	
Group	*n*	*n*	%
Control (0-βME)	65	31	47.7 ± 1.8^{b}
50-βME	63	33	$52.4 \pm 1.3^{a,b}$
100-βME	63	42	66.7 ± 1.2^{a}

^{a,b}Numbers in the column with distinct superscripts differ ($P < 0.05$).

(52.4%). Based on such results, after five replications, the 100 μM βME concentration was chosen to be used in Experiment II.

Experiment II: Effect of βME on embryo cryotolerance and post-cryopreservation viability

Results summarizing the effects of 100 μM βME supplementation during the IVC and/or the PWC are presented in Table 2 and Figures 1–4. In total, 3735 COCs used for bovine IVP of embryos, and after seven replications, the supplementation of 100 μM βME to the IVC did not affect cleavage rates on Day 2 of development, which averaged 80.6%. However, compared with the control group, βME-IVC negatively interfered with subsequent embryo development to the blastocyst stage (Table 2) and caused a delay in embryo kinetics on Day 7 of development (Figure 2). Compared with the control, βME-IVC resulted in a greater proportion of embryos on stages 5 and 6, and a smaller proportion of embryos on stages 7–9 (Figure 2). Embryo quality classification, however, was not affected by treatment (Table 2).

Data on the effect of βME on embryo re-expansion and hatching rates in all groups are presented in Table 3 and Figure 3. The use of βME during IVC, both prior to (βME–CTRL), and prior to and after (βME–βME) vitrification of Day-7 blastocysts increased re-expansion rates compared with the CTRL–CTRL group. However, re-expansion rates in the CTRL–βME group were lower than the βME–βME group and did not differ from the CTRL–CTRL and the βME–CTRL groups. Compared with the CTRL–CTRL group, total hatching rates were higher after βME supplementation after (CTRL–βME), and prior to and after (βME–βME) vitrification. Supplementation of βME during the IVC period alone (βME–CTRL) resulted in similar hatching rates to CTRL–CTRL, which was also lower compared with the CTRL–βME and βME–βME groups (Table 3). On a daily basis, such differences were pronounced only 48 h after warming, as shown in Figure 3(a). When the main effect of βME supplementation in IVC was evaluated (CTRL–CTRL + CTRL–βME vs. βME–CTRL + βME–βME), βME supplementation improved the rate of blastocyst re-expansion but did not affect the rate of blastocyst hatching after vitrification (Figure 3b). The opposite was seen for the main effect of βME supplementation in PWC (CTRL–CTRL + βME–CTRL vs. CTRL–βME + βME–βME), in which βME supplementation did not affect the rate of blastocyst re-expansion but enhanced the rate of blastocyst hatching after vitrification (Figure 3c).

As for total cell number in hatched blastocysts, those from βME–βME group had a greater number of cells than those in CTRL–CTRL (181.2 ± 7.4 vs. 139.0 ± 9.9 cells, respectively), with the total cell counts in embryos in CTRL–βME (165.2 ± 6.7 cells) and in the βME–CTRL (159.9 ± 9.2 cells) groups being intermediate and similar to all groups (Figure 4a). Regarding the main effects of the factorial treatments, a greater number of cells in hatched blastocysts was observed for βME supplementation in both IVC (Figure 4b) and PWC (Figure 4c).

Discussion

This study evaluated the effects of adding βME in the IVC medium on the development, quality, viability, and cryotolerance of bovine embryos prior to and after vitrification. Although the effects of supplementing IVC medium with βME on the developmental ability and quality of IVP mammalian bovine embryos have been examined previously (Takahashi *et al.*, 1993, 1996; Caamaño *et al.*, 1998; de Matos and Furnus, 2000; Feugang *et al.*, 2004; Mori *et al.*, 2006; Nedambale *et al.*, 2006; Moussa *et al.*, 2019), no previous study has compared the effect of βME supplementation both prior to and after the vitrification process on the viability of IVP embryos. Therefore, we tested the hypothesis that βME added to the culture medium of bovine embryos, before and/or after cryopreservation, would increase the cryotolerance to vitrification. Our results showed that adding 100 μM βME in the IVC period improved cryotolerance, as indicated by increased re-expansion rates and total cell number of hatched blastocysts after vitrification, whereas adding 100 μM βME in the PWC increased hatching rates and total cell number of hatched blastocysts after vitrification.

Initially we conducted a dose–response experiment to determine an optimal βME concentration in PWC conditions because of the large variation in concentrations reported in the literature, which ranged from 5 μM to 500 μM (Takahashi *et al.*, 1993; de Matos *et al.*, 1996; Caamaño *et al.*, 1998; Geshi *et al.*, 1999; Feugang *et al.*, 2004). Although the supplementation with 50 μM or 100 μM βME had no significant differences on hatching rates between one another in Experiment I, 100 μM βME promoted higher hatching rates than controls (0 μM βME), corroborating with previous studies that demonstrated that 100 μM supported adequate embryo development (Caamaño *et al.*, 1998; Nedambale *et al.*, 2006; Hosseini *et al.*, 2009).

In general, data on the effects of βME on embryo development are inconsistent regarding the optimal concentration of βME used in the culture medium (Hosseini *et al.*, 2009). Geshi *et al.* (1999) showed that 10 μM βME in a co-culture system improved embryo development. Caamaño *et al.* (1998) noted that 10 and 100 μM of βME in the culture medium had no difference in blastocyst development (25.3% and 21.3%, respectively), but blastocyst rates in both groups were higher than in the controls without the GSH precursor (10.7%). In contrast, supplementation of 100 μM βME resulted in lower development rates of bovine embryos compared with 50 μM βME (Mori *et al.*, 2006). The latter corroborated with Takahashi *et al.* (1993), who reported that lower concentrations of βME (e.g. 50 μM) promoted better embryo development than higher concentrations, which had detrimental effects on the hatching capacity of embryos. Interestingly, Feugang *et al.* (2004) showed that 50 μM in the culture medium had no protective effects on embryos, but supplementation with 100 μM βME increased hatching rates and total cell numbers in surviving blastocysts. In the same study, 100 μM βME reduced apoptosis and stimulated glutathione synthesis in Day-7 IVP embryos. Conversely, studies in other species, such as the pig, demonstrated that 50 μM and 100 μM βME supplementation had no effect on embryo development, total cell number, apoptosis index, and cryotolerance (Castillo-Martín *et al.*, 2015). According to Hosseini *et al.*

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

Table 2. Cleavage and blastocyst rates, and embryo quality by morphological evaluation after *in vitro* culture (IVC) of bovine IVP embryos in IVC medium supplemented with 0 (CTRL–IVC) or 100 µM βME (βME–IVC)

Group	Embryos in IVC	Cleavage rate		Blastocyst rate		Embryo quality (%)	
	n	*n*	%	*n*	%	Grade 1	Grade 2
CTRL–IVC	1858	1491	80.2 ± 0.9	813	43.8 ± 1.3[a]	65.8 ± 1.0	34.2 ± 1.0
βME–IVC	1877	1522	81.1 ± 0.8	525	28.0 ± 2.9[b]	68.9 ± 0.9	31.1 ± 0.9

[a,b]Numbers in the same column with distinct superscripts differ ($P < 0.05$).

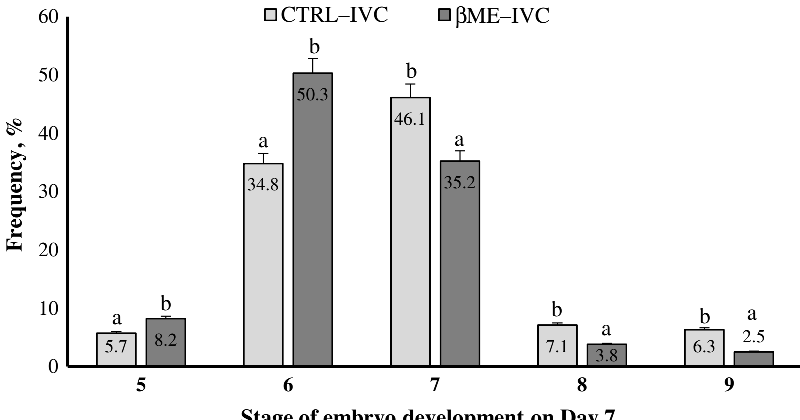


Figure 2. Developmental kinetics of Day-7 bovine blastocysts after IVC from Day 1 to Day 7 in medium supplemented (βME–IVC) or not (CTRL–IVC) with 100 µM βME. Classification of stage of development: 5 = early blastocyst; 6 = blastocyst; 7 = expanded blastocyst; 8 = hatching blastocyst; 9 = hatched blastocyst. [a,b]Within stage of development, columns with distinct superscripts differ ($P < 0.05$).

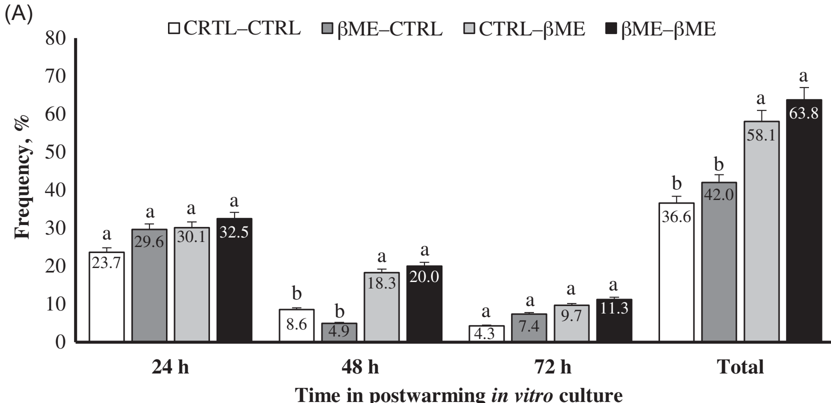
(2009), the apparent inconsistency among studies could be attributed to the culture conditions or developmental stages in which βME has been added. In fact, differential effects of βME as an antioxidant on embryo development seem to be dependent on the species, breed, supplementation dose, cell type, and bioavailability on the culture medium (Nikseresht *et al.*, 2017). Although apoptosis was not evaluated in our study, the use of βME in the PWC medium had beneficial effects by increasing embryo survival, manifested by higher hatching rates and total cell numbers in hatched blastocysts.

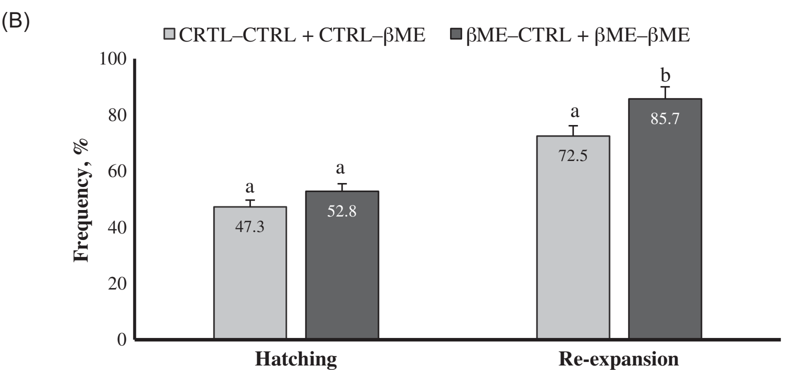
Zona pellucida (ZP) thickness is considered a morphological marker associated with the likelihood of hatching and implantation outcomes in transferred frozen–thawed embryos (Balakier *et al.*, 2012; Nada *et al.*, 2018). Although ZP thickness of blastocysts was not measured in our study, higher hatching rates under the 100-µM βME supplementation might be related to structural changes in the ZP mediated by thiol-disulfide exchange reactions, in which the thiol group reduces disulfide bonds and induces expansion in ZP glycoproteins (Takeo *et al.*, 2015; Truong and Gardner, 2017). Nonetheless, the higher total cell numbers of blastocysts supplemented with βME indicated improvements in cell proliferation that could lead to faster growth and expansion of blastocysts, promoting mechanical thinning of the ZP and successful hatching of the blastocyst (Goud *et al.*, 2014; Khanmohammadi *et al.*, 2016; Giorgi *et al.*, 2021), a phenomenon already reported by others that tested supplementation with the antioxidant *N*-acetylcysteine (Giorgi *et al.*, 2021).

Results from Experiment II indicated that the 100 µM βME supplementation during the IVC had no influence on cleavage rates (80.2% for controls vs. 81.1% for βME), noting that the addition of βME only on Day 1 was not enough to interfere with the first cleavage. In similar experimental designs, Hosseini *et al.* (2009) and Rocha-Frigoni *et al.* (2014) also found no interference from βME in cleavage rates. However, in contrast with our findings, results reported by Hosseini *et al.* (2009) indicated that βME supplementation in the IVC medium, either during 1–8 days and/or 9–10 days of embryo culture, improved the overall developmental competence and quality of bovine IVP embryos. Such findings are consistent with those reported for 4-cell and 8-cell (Geshi *et al.*, 1999) and 8-cell to 16-cell (Caamaño *et al.*, 1998) stage embryos, and beyond the morula stage (Feugang *et al.*, 2003).

Healthy *in vitro* embryo development depends on keeping the redox homeostasis of blastomeres, which requires a balance between ROS and antioxidant compounds (Bain *et al.*, 2011; Jamil *et al.*, 2020; Lin and Wang, 2020). Low or moderate levels of ROS are critical for the signal transduction for certain biologically active factors such as nuclear factor κB (NF-κB) (Jamil *et al.*, 2020), which are associated with the regulation of maternal-to-embryonic transition and cell differentiation (Schreck *et al.*, 1991). In this study, 100 µM βME during IVC interfered negatively with blastocyst rates and developmental kinetics. Tsuzuki *et al.* (2005) suggested that βME may remove some divalent cations that are needed for embryonic development prior to the 2-cell stage. Similar results were reported by Rocha-Frigoni *et al.* (2014) who obtained reduced blastocyst rates in bovine embryos supplemented with 100 µM βME compared with unsupplemented controls (33.4% vs. 48.7%, respectively). In agreement with our findings, it is possible that higher βME concentrations (100 µM) during IVC result in suboptimal ROS levels and negatively affect important cellular processes such as the NF-κB signalling. This concept is supported by evidence suggesting that fluctuations in the oxidants: antioxidants ratio should affect first bovine embryo cleavage, and also affect embryo kinetics and development to the blastocyst stage (Lopes *et al.*, 2010).

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press





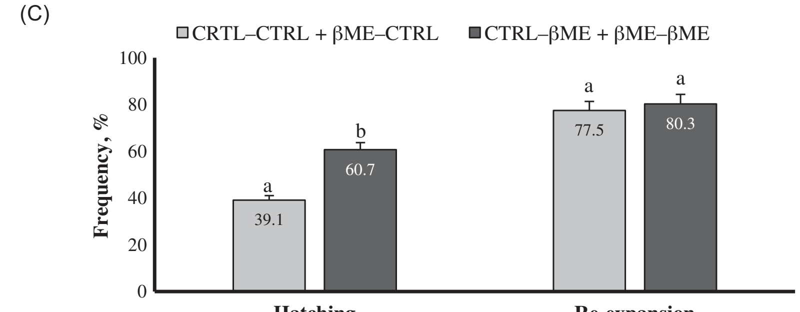


Figure 3. Effect of 100 μM β-Mercaptoethanol (βME) added to *in vitro* culture (IVC) and/or post-warming culture (PWC) medium on daily blastocyst hatching rates through 72 h after vitrification according to factorial arrangement of treatments: CTRL–CTRL: control IVC and control PWC; CTRL–βME: control IVC and βME-supplemented PWC; βME–CTRL: βME-supplemented IVC and control PWC; βME–βME: βME-supplemented IVC and βME-supplemented PWC (A). Blastocyst hatching rates at 72 h according to main effects of βME on IVC (B) and the main effects of βME on PWC (C). [a,b]Within time of evaluation, columns with distinct superscripts differ ($P < 0.05$).

Despite the negative effects of βME supplementation during IVC observed in our study, most studies in the literature have demonstrated improvements in embryo development with supplementation of different βME doses in the culture medium (Takahashi *et al.*, 1993; Caamaño *et al.*, 1998; Geshi *et al.*, 1999; Hosseini *et al.*, 2009). The inconsistency of our findings in comparison with those reported by others may be related to the fact that blastocyst rates in the control group were rather low in those studies (7.1%, 10.7%, 15.4%, and 17.1%, respectively), which suggests that βME supplementation during IVC may be beneficial under unfavourable culture conditions that result in redox unbalance, becoming less helpful when blastocyst rates exceed 30–40% (Rizos *et al.*, 2002), as seen in this study.

Vitrification can impair the developmental competence of embryos (Moussa *et al.*, 2019; Leme *et al.*, 2020) and this effect can be evaluated by embryo developmental kinetics (Leme *et al.*, 2020). In the current study, despite the reduction in blastocyst development by Day 7, embryos supplemented with βME during IVC had improved cryotolerance to vitrification based on the increased rate of re-expansion after warming (βME-IVC = 85.7 vs. Control = 72.5). In addition, supplementation with βME in the PWC medium showed a significant increase in blastocyst hatching rates during the first 72 h of post-warming culture (βME-IVC = 60.7 vs. Control = 39.1%). Despite the independent results of βME supplementation in IVC and in PWC, the combination of βME supplementation in both IVC and PWC further enhanced cryotolerance of blastocysts to vitrification, resulting in an 87.5% re-expansion and 63.8% hatching. Furthermore, hatched blastocysts exposed to βME before or after vitrification had greater number of cells at 72 h post-warming compared with controls, which further supported the positive effects of βME in cryotolerance to vitrification. In contrast with our study, Hosseini *et al.* (2009) reported no differences in re-expansion rates when supplementing culture medium with 100 μM βME prior to vitrification (Days 1–7 of IVC). Nevertheless, Nedambale *et al.* (2006) demonstrated that supplementation with 100 μM βME to the medium after blastocyst vitrification and warming significantly increased blastocyst re-expansion and survival for the first 6 h, hatching rates, and total cell numbers. Similar effects were obtained in hatched bovine blastocysts on Day 8 (Feugang *et al.*, 2004) and on Day 9 (Van Soom *et al.*, 2002) when embryos were cultured with βME and cysteine, respectively. These findings suggest that the viability of

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

Table 3. Re-expansion and hatching of vitrified bovine blastocysts produced *in vitro* supplemented with 0 or 100 µM of βME during the *in vitro* culture (IVC) and/or during the post-warming culture (PWC)

Experimental group[1]	Embryos in culture after vitrification *n*	Re-expansion rate[2]		Total hatching rate[2]	
		n	%	*n*	%
CTRL–CTRL	93	66	71.0 ± 1.9[a]	34	36.6 ± 5.1[a]
βME–CTRL	81	68	84.0 ± 0.4[bc]	34	42.0 ± 3.3[a]
CTRL–βME	93	68	73.1 ± 1.4[ab]	54	58.1 ± 2.6[b]
βME–βME	80	70	87.5 ± 1.0[c]	51	63.8 ± 1.1[b]

[a,b,c]Numbers in the same column with distinct superscripts differ ($P < 0.05$).
[1]Experimental group: CTRL–CTRL = control IVC and control PWC; CTRL–βME = control IVC and βME-supplemented PWC; βME–CTRL = βME-supplemented IVC and control PWC; βME–βME = βME-supplemented IVC and βME-supplemented PWC.
[2]Re-expansion was evaluated at 12 h and total hatching was evaluated at 72 h after rewarming of vitrified bovine blastocysts.

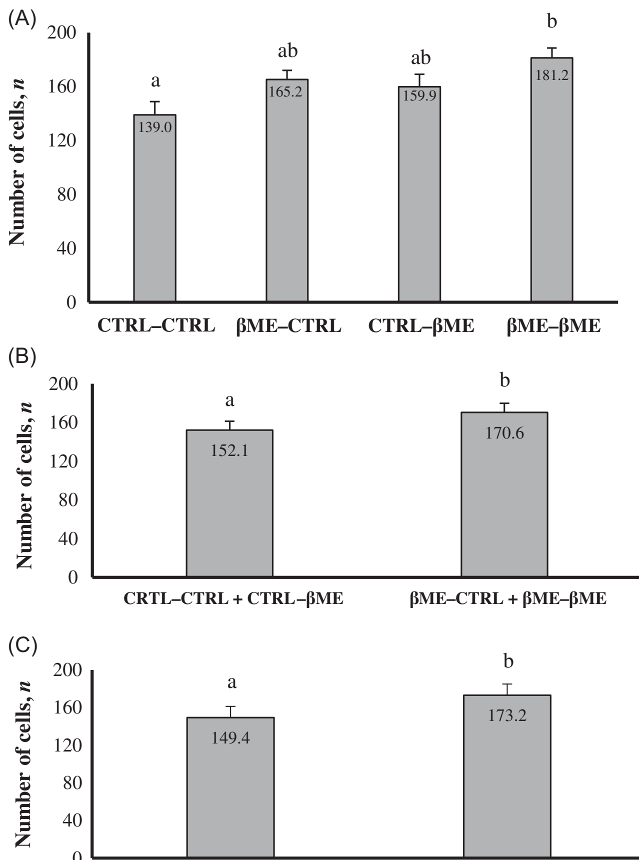


Figure 4. Effect of 100 µM β-mercaptoethanol (βME) added to *in vitro* culture (IVC) and/or post-warming culture (PWC) medium on total cell number in hatched blastocysts 72 h after vitrification according to factorial arrangement of treatments. CTRL–CTRL: control IVC and control PWC; CTRL–βME: control IVC and βME-supplemented PWC; βME–CTRL: βME-supplemented IVC and control PWC; βME–βME: βME-supplemented IVC and βME-supplemented PWC (A). Total cell number in hatched blastocysts according to the main effects of βME on IVC (B) and the main effects of βME on PWC (C). [a,b]Within time of evaluation, columns with distinct superscripts differ ($P < 0.05$).

vitrified blastocysts can be improved if warmed and briefly cultured in medium supplemented with antioxidants prior to embryo transfer.

From a biochemical point of view, βME is a low-molecular-weight thiol that interacts directly with a few oxidized radicals (Mori *et al.*, 2006) and acts as a precursor to L-gamma-glutamyl-L-cysteinyl-glycine, also known as glutathione (GSH). Thiols protect cysteine from oxidation by reducing disulfide bridges in cysteine molecules, and increase its absorption by cells, thus promoting GSH synthesis (Takahashi *et al.*, 1993; Feugang *et al.*, 2004; Rashidipour *et al.*, 2020). Glutathione is considered the major representative of nonenzymatic antioxidants present in oocytes and embryos (Rodríguez-González *et al.*, 2003) and it is associated with the removal of ROS and detoxification of lipid peroxides (Johnson and Nasr-Esfahani, 1994). These aspects make GSH interesting for IVP embryos because oocytes and embryos cultured *in vitro* accumulate more lipids than *in vivo*-derived embryos (Rizos *et al.*, 2002). Accumulation of ROS and intracellular lipids during IVP of embryos increases the peroxidation of cell membranes (de Matos *et al.*, 1996), and the cellular damage caused by cryopreservation makes cells even more susceptible to the actions of ROS (Agarwal *et al.*, 2006). Although GSH levels in embryos were not evaluated in our study, other reports have demonstrated that βME supplementation during IVC increases intracellular GSH levels and blastocyst rates (Takahashi *et al.*, 1993) and reduces lipid peroxidation (de Matos *et al.*, 1996). In another study, βME-induced GSH provided an available reserve until the first cleavage, improving the IVP efficiency of embryos from immature oocytes (de Matos and Furnus, 2000). Despite the known role that GSH plays in the maintenance of the intracellular redox state (Feugang *et al.*, 2004), the mechanism of action by which βME exerts its effect on embryos is still not completely elucidated.

In addition to βME, several other substances of distinct natures and mechanisms of actions have been proposed as antioxidant supplements to medium for the culture and cryopreservation of somatic cells and *in vitro* embryo production in humans and animals, both under experimental and clinical protocols (Budani and Tiboni, 2020). To note, some examples include the use of resveratrol, melatonin, coenzyme Q, Vitamins (A, B complex, C, D and E), acetyl-L-carnitine, *N*-acetyl-cysteine, α-lipoic acid and, similar to βME, thiol compounds that stimulate GSH synthesis, such as dithiothreitol (DTT), and cysteamine. Similar to βME, and as a small molecule redox reagent used to reduce disulfide bonds and maintain monothiols in a reduced state (Cleland, 1964), DTT has proved to be beneficial for mouse and human oocytes and embryos when in culture, perhaps with less potentially toxicity than βME (Cleland, 1964; Tarín *et al.*, 1998; Liu *et al.*, 1999). We are unaware of studies focusing on the toxic levels of βME on mammalian embryos. But it is important to mention that an excess of thiol compounds may impair the redox equilibrium during oocyte maturation and subsequent embryo development (Guérin *et al.*, 2001). Liu *et al.* (1999) showed that altering the thiol redox status in mouse embryos

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

induced cell cycle arrest and cell death. In this way, Tsuzuki *et al.* (2005), exploring the effects of βME during IVM and IVC on bovine IVP, showed that 100 μM βME increased both the numbers of cumulus cells attached to oocytes, as well as the total cell numbers in blastocysts by modulating ATP metabolism in oocytes. In contrast, 500 μM βME decreased embryo development, which suggests that concentrations of more than 100 μM βME could be detrimental to bovine embryos. More studies are needed to clarify the possible βME toxic effects on mammalian embryo development *in vitro*.

It is generally believed that βME supplementation in culture medium improves the developmental competence of IVP embryos, maintaining the levels of intracellular glutathione and improving *in vivo* development following transfer to female recipients (Truong *et al.*, 2016). Similarly, βME addition to *in vitro* culture medium prior to (Takahashi *et al.*, 1996; Caamaño *et al.*, 1998) and after vitrification (Nedambale *et al.*, 2006) has been shown to improve vitrified blastocyst survival rate, incubation capacity, total cells numbers, and the protection against apoptosis. In fact, findings by Moussa *et al.* (2019) demonstrated a higher level of expression of E-cadherin, β-catenin, and Oct4 in vitrified blastocysts cultured in the presence of βME, suggesting that βME protects vitrified blastocyst against apoptosis. In turn, vitrified embryos in the absence of βME showed a reduction in the expression of E-cadherin and β-catenin, also causing significant changes in the expression of Oct4, Cdx2, and Gata3, which are essential factors for the development of blastocysts and cell differentiation into inner cell mass (ICM) and trophectoderm (TE) cells in bovine embryos (Goissis and Cibelli, 2014; Sakurai *et al.*, 2016). E-cadherin is a transmembrane surface molecule that involves Ca^{2+}-dependent cells for cellular adhesion to other cells (Takeichi, 1988), which is related to compaction (Fleming *et al.*, 1984). E-cadherin transcripts and protein were found in both the ICM and TE of expanded bovine blastocysts (Barcroft *et al.*, 1998). The suppression of E-cadherin mRNA and protein resulted in lower blastocyst rates (Nganvongpanit *et al.*, 2006). β-Catenin is related to cell-to-cell adhesion, acting as an intracellular signalling molecule from the cytoplasm to the nucleus (Willert and Nusse, 1998). Oct4 plays a role as an anti-apoptotic factor (Guo *et al.*, 2008). Therefore, decreased expression of Oct4 may disturb the balance between pro-apoptotic and anti-apoptotic factors, which in turn may lead to a higher frequency of apoptotic cells (Nedambale *et al.*, 2006). Thus, βME seems to play a critical role in increasing the resistance of vitrified embryos to oxidative stress, reducing apoptosis.

In our study, the observed detrimental effects of adding βME in the 6-d IVC and the beneficial effects of adding βME on the 3-d PWC were likely to have been mediated by the activity of βME or its metabolites during the first day of culture and exposure to treatment. Although βME is highly stable in aqueous solution (Wong *et al.*, 2014), Stevens *et al.* (1983) has shown that βME has a short half-life time, depending on the pH and temperature of the medium (~10 h at pH 7.5 and at 20°C). Therefore, considering that we did not replenish the culture medium with additional βME during both culture periods, IVC and PWC, it is likely that βME bioavailability was not constant during the entire duration of the incubation periods and the effects were mediated towards the beginning of the incubation periods, when bioavailability of βME was greater. In the IVC period, specifically, embryos were cultured in 5% CO_2 and ~20% O_2 in the first 24 h, and then placed in an atmosphere of 90% N_2, 5% O_2 and 5% CO_2 after evaluation of cleavage rates on Day 2 until Day 7 and after warming of vitrified blastocysts. Thus, the βME treatment was likely to be more active in the first 24 h of IVC, when oxygen tension was higher and when the formation of free radicals was more likely to occur (de Castro e Paula and Hansen, 2008). In the PWC culture, the βME treatment was likely to be more active in the first few hours after warming of vitrified blastocysts, when the oxidative stress associated with cryo-injuries is more substantial (Deleuze and Goudet, 2010; Truong and Gardner, 2020). Therefore, although we did not replenish βME in the culture medium to ensure that its bioavailability was more constant throughout the culture periods, we believe that the most critical points of oxidative stress in our IVP/freezing model were covered with antioxidant supplementation. Interestingly, despite the short half-life, the manifestation of biological effects of βME seemed to persist for several days. In fact, we did not observe an effect of βME in the cleavage rate on Day 2, but observed a large difference in blastocyst development on Day 7 and important changes in blastocyst cryotolerance up to Day 10. In the PWC, the βME treatment affected expansion rate in the first 24 h, but the effects on blastocyst hatching seemed to be maintained until 72 h post-warming.

In summary, our study indicated that supplementing culture medium with 100 μM βME prior to and/or after vitrification improved survival and developmental rates of bovine embryos following vitrification. An increase in cryotolerance rates was observed, with higher survival and hatching rates, better quality blastocysts, especially when βME was supplemented after warming. Even though βME supplementation during IVC reduced blastocyst yield and delayed embryo kinetics, the resulting blastocysts had greater re-expansion rates following vitrification and warming. βME supplementation during *in vitro* PWC, in combination or not with its use during *in vitro* culture prior to vitrification, increased embryo survival rates after cryopreservation, and increasing hatching rates and total cell numbers in hatched blastocysts. The positive effect of βME on cryotolerance can be maximized if added throughout the culture period. Future investigations must be performed to elucidate the effect of βME at the molecular, metabolic, and physicochemical levels on bovine IVP embryos in order to understand the mechanisms related to the acquisition of cryoresistance in embryos, and to determine the optimal dose–response effects of βME supplementation during IVC to optimize embryo development in different IVP conditions.

Acknowledgements. The authors thank Frigorífico Verdi Ltda., from Pouso Redondo, SC, Brazil, for supplying bovine ovaries.

Financial support. This study was partially supported by research grant no. 0192058 from CAPES/Brazil. K.M. was supported by a scholarship from CAPES/Brazil.

Conflict of interest. The authors declare none.

Ethics statements. Not applicable.

References

Abe, H., Yamashita, S., Satoh, T. and Hoshi, H. (2002). Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Molecular Reproduction and Development*, **61**(1), 57–66. doi: 10.1002/mrd.1131

Agarwal, A., Sharma, R. K., Nallella, K. P., Thomas, A. J., Alvarez, J. G. and Sikka, S. C. (2006). Reactive oxygen species as an independent marker of

male factor infertility. *Fertility and Sterility*, **86**(4), 878–885. doi: 10.1016/j.fertnstert.2006.02.111

Bain, N. T., Madan, P. and Betts, D. H. (2011). The early embryo response to intracellular reactive oxygen species is developmentally regulated. *Reproduction, Fertility, and Development*, **23**(4), 561–575. doi: 10.1071/RD10148

Balakier, H., Sojecki, A., Motamedi, G., Bashar, S., Mandel, R. and Librach, C. (2012). Is the zona pellucida thickness of human embryos influenced by women's age and hormonal levels? *Fertility and Sterility*, **98**(1), 77–83. doi: 10.1016/j.fertnstert.2012.04.015

Barcroft, L. C., Hay-Schmidt, A., Caveney, A., Gilfoyle, E., Overstrom, E. W., Hyttel, P. and Watson, A. J. (1998). Trophectoderm differentiation in the bovine embryo: Characterization of a polarized epithelium. *Journal of Reproduction and Fertility*, **114**(2), 327–339. doi: 10.1530/jrf.0.1140327

Budani, M. C. and Tiboni, G. M. (2020). Effects of supplementation with natural antioxidants on oocytes and preimplantation embryos. *Antioxidants*, **9**(7), 612–637. doi: 10.3390/antiox9070612

Caamaño, J. N., Ryoo, Z. Y. and Youngs, C. R. (1998). Promotion of development of bovine embryos produced in vitro by addition of cysteine and β-mercaptoethanol to a chemically defined culture system. *Journal of Dairy Science*, **81**(2), 369–374. doi: 10.3168/jds.S0022-0302(98)75586-9

Castillo-Martín, M., Yeste, M., Pericuesta, E., Morató, R., Gutiérrez-Adán, A. and Bonet, S. (2015). Effects of vitrification on the expression of pluripotency, apoptotic and stress genes in *in vitro*-produced porcine blastocysts. *Reproduction, Fertility, and Development*, **27**(7), 1072–1081. doi: 10.1071/RD13405

Cleland, W. W. (1964). Dithiothreitol a new protective reagent for SH groups. *Biochemistry*, **3**, 480–482. doi: 10.1021/bi00892a002

Cornell, N. W. and Crivaro, K. E. (1972). Stability constant for the zinc-dithiothreitol complex. *Analytical Biochemistry*, **47**, 203–208. doi: 10.1016/0003-2697(72)90293-x

de Castro e Paula, L. A. and Hansen, P. J. (2008). Modification of actions of heat shock on development and apoptosis of cultured preimplantation bovine embryos by oxygen concentration and dithiothreitol. *Molecular Reproduction and Development*, **75**(8), 1338–1350. doi: 10.1002/mrd.20866

de Matos, D. G. and Furnus, C. C. (2000). The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: Effect of b-mercaptoethanol, cysteine and cystine. *Theriogenology*, **53**(3), 761–771. doi: 10.1016/S0093-691X(99)00278-2

de Matos, D. G., Furnus, C. C., Moses, D. F., Martinez, A. G. and Matkovic, M. (1996). Stimulation of glutathione synthesis of *in vitro* matured bovine oocytes and its effect on embryo development and freezability. *Molecular Reproduction and Development*, **45**(4), 451–457. doi: 10.1002/(SICI)1098-2795(199612)45:4<451::AID-MRD7>3.0.CO;2-Q

Deleuze, S. and Goudet, G. (2010). Cysteamine supplementation of *in vitro* maturation media: A review. *Reproduction in Domestic Animals*, **45**(6), e476–e482. doi: 10.1111/j.1439-0531.2010.01587.x

Feugang, J. M., Van Langendonckt, A., Sayoud, H., Rees, J. F., Pampfer, S., Moens, A., Dessy, F. and Donnay, I. (2003). Effect of prooxidant agents added at the morula/blastocyst stage on bovine embryo development, cell death and glutathione content. *Zygote*, **11**(2), 107–118. doi: 10.1017/s0967199403002144

Feugang, J. M., de Roover, R., Moens, A., Léonard, S., Dessy, F. and Donnay, I. (2004). Addition of β-mercaptoethanol or Trolox® at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. *Theriogenology*, **61**(1), 71–90. doi: 10.1016/S0093-691X(03)00191-2

Fleming, T. P., Warren, P. D., Chisholm, J. C. and Johnson, M. H. (1984). Trophectodermal processes regulate the expression of totipotency within the inner cell mass of the mouse expanding blastocyst. *Journal of Embryology and Experimental Morphology*, **84**, 63–90. doi: 10.1242/dev.84.1.63

Geshi, M., Yonai, M., Sakaguchi, M. and Nagai, T. (1999). Improvement of *in vitro* co-culture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with beta-mercaptoethanol. *Theriogenology*, **51**(3), 551–558. doi: 10.1016/s0093-691x(99)00009-6

Giorgi, V. S. I., Ferriani, R. A. and Navarro, P. A. (2021). Follicular fluid from infertile women with mild endometriosis impairs *in vitro* bovine embryo development: Potential role of oxidative stress. *Revista Brasileira de Ginecologia e Obstetrícia: revista da Federação Brasileira das Sociedades de Ginecologia e Obstetrícia*, **43**(2), 119–125. doi: 10.1055/s-0040-1718443

Goissis, M. D. and Cibelli, J. B. (2014). Functional characterization of CDX2 during bovine preimplantation development *in vitro*. *Molecular Reproduction and Development*, **81**(10), 962–970. doi: 10.1002/mrd.22415

Gómez, E., Carrocera, S., Martín, D., Pérez-Jánez, J. J., Prendes, J., Prendes, J. M., Vázquez, A., Murillo, A., Gimeno, I. and Muñoz, M. (2020). Efficient one-step direct transfer to recipients of thawed bovine embryos cultured in vitro and frozen in chemically defined medium. *Theriogenology*, **146**, 39–47. doi: 10.1016/j.theriogenology.2020.01.056

Goud, P. T., Goud, A. P., Joshi, N., Puscheck, E., Diamond, M. P. and Abu-Soud, H. M. (2014). Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis. *Fertility and Sterility*, **102**(1), 151–159.e5. doi: 10.1016/j.fertnstert.2014.03.053

Guérin, P., El Mouatassim, S. and Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update*, **7**(2), 175–189. doi: 10.1093/humupd/7.2.175

Guo, Y., Mantel, C., Hromas, R. A. and Broxmeyer, H. E. (2008). Oct-4 is critical for survival/antiapoptosis of murine embryonic stem cells subjected to stress: Effects associated with Stat3/survivin. *Stem Cells*, **26**(1), 30–34. doi: 10.1634/stemcells.2007-0401

Holm, P., Booth, P. J., Schmidt, M. H., Greve, T. and Callesen, H. (1999). High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum proteins. *Theriogenology*, **52**(4), 683–700. doi: 10.1016/S0093-691X(99)00162-4

Hosseini, S. M., Forouzanfar, M., Hajian, M., Asgari, V., Abedi, P., Hosseini, L., Ostadhosseini, S., Moulavi, F., Safahani Langrroodi, M., Sadeghi, H., Bahramian, H., Eghbalsaied, Sh and Nasr-Esfahani, M. H. (2009). Antioxidant supplementation of culture medium during embryo development and/or after vitrification-warming; which is the most important? *Journal of Assisted Reproduction and Genetics*, **26**(6), 355–364. doi: 10.1007/s10815-009-9317-7

Jamil, M., Debbarh, H., Aboulmaouahib, S., Aniq Filali, O., Mounaji, K., Zarqaoui, M., Saadani, B., Louanjli, N. and Cadi, R. (2020). Reactive oxygen species in reproduction: harmful, essential or both? *Zygote*, **28**(4), 255–269. doi: 10.1017/S0967199420000179

Johnson, M. H. and Nasr-Esfahani, M. H. (1994). Radical solutions and cultural problems: Could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, **16**(1), 31–38. doi: 10.1002/bies.950160105

Khanmohammadi, N., Movahedin, M., Safari, M., Sameni, H. R., Yousefi, B., Jafari, B. and Zarbakhsh, S. (2016). Effect of L-carnitine on in vitro developmental rate, the zona pellucida and hatching of blastocysts and their cell numbers in mouse embryos. *International Journal of Reproductive Biomedicine*, **14**(10), 649–656. doi: 10.29252/ijrm.14.10.649

Leme, L. O., Carvalho, J. O., Franco, M. M. and Dode, M. A. N. (2020). Effect of sex on cryotolerance of bovine embryos produced *in vitro*. *Theriogenology*, **141**, 219–227. doi: 10.1016/j.theriogenology.2019.05.002

Lin, J. and Wang, L. (2020). Oxidative stress in oocytes and embryo development: Implications for *in vitro* systems. *Antioxidants and Redox Signaling*, **34**, 1394–1406. doi: 10.1089/ars.2020.8209

Liu, L., Trimarchi, J. R. and Keefe, D. L. (1999). Thiol oxidation-induced embryonic cell death in mice is prevented by the antioxidant dithiothreitol. *Biology of Reproduction*, **61**(4), 1162–1169. doi: 10.1095/biolreprod61.4.1162

Lonergan, P., Rizos, D., Gutierrez-Adan, A., Fair, T. and Boland, M. P. (2003). Oocyte and embryo quality: Effect of origin, culture conditions and gene expression patterns. *Reproduction in Domestic Animals*, **38**(4), 259–267. doi: 10.1046/j.1439-0531.2003.00437.x

Lopes, A. S., Lane, M. and Thompson, J. G. (2010). Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

bovine zygotes. *Human Reproduction*, **25**(11), 2762–2773. doi: 10.1093/humrep/deq221
Mori, M., Otoi, T., Wongsrikeao, P., Agung, B. and Nagai, T. (2006). Effects of beta-mercaptoethanol and cycloheximide on survival and DNA damage of bovine embryos stored at 4°C for 72 h. *Theriogenology*, **65**(7), 1322–1332. doi: 10.1016/j.theriogenology.2005.07.018
Moussa, M., Yang, C. Y., Zheng, H. Y., Li, M. Q., Yu, N. Q., Yan, S. F., Huang, J. X. and Shang, J. H. (2019). Vitrification alters cell adhesion related genes in pre-implantation buffalo embryos: Protective role of β-mercaptoethanol. *Theriogenology*, **125**, 317–323. doi: 10.1016/j.theriogenology.2018.11.013
Nada, A. M., El-Noury, A., Al-Inany, H., Bibars, M., Taha, T., Salama, S., Hassan, F. and Zein, E. (2018). Effect of laser-assisted zona thinning, during assisted reproduction, on pregnancy outcome in women with endometriosis: Randomized controlled trial. *Archives of Gynecology and Obstetrics*, **297**(2), 521–528. doi: 10.1007/s00404-017-4604-5
Nedambale, T. L., Du, F., Yang, X. and Tian, X. C. (2006). Higher survival rate of vitrified and thawed *in vitro* produced bovine blastocysts following culture in defined medium supplemented with β-mercaptoethanol. *Animal Reproduction Science*, **93**(1–2), 61–75. doi: 10.1016/j.anireprosci.2005.06.027
Nganvongpanit, K., Müller, H., Rings, F., Gilles, M., Jennen, D., Hölker, M., Tholen, E., Schellander, K. and Tesfaye, D. (2006). Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. *Molecular Reproduction and Development*, **73**(2), 153–163. doi: 10.1002/mrd.20406
Nikseresht, M., Toori, M. A., Rahimi, H. R., Fallahzadeh, A. R., Kahshani, I. R., Hashemi, S. F., Bahrami, S. and Mahmoudi, R. (2017). Effect of antioxidants (β-mercaptoethanol and cysteamine) on assisted reproductive technology *in vitro*. *Journal of Clinical and Diagnostic Research*, **11**(2), BC10–BC14. doi: 10.7860/JCDR/2017/21778.9298
Rashidipour, N., Karami-Mohajeri, S., Mandegary, A., Mohammadinejad, R., Wong, A., Mohit, M., Salehi, J., Ashrafizadeh, M., Najafi, A. and Abiri, A. (2020). Where ferroptosis inhibitors and paraquat detoxification mechanisms intersect, exploring possible treatment strategies. *Toxicology*, **433–434**, 152407. doi: 10.1016/j.tox.2020.152407
Ray, P. D., Huang, B. W. and Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signaling*, **24**(5), 981–990. doi: 10.1016/j.cellsig.2012.01.008
Ribeiro, E. S., Gerger, R. P., Ohlweiler, L. U., Ortigari, I., Jr., Mezzalira, J. C., Forell, F., Bertolini, L. R., Rodrigues, J. L., Ambrósio, C. E., Miglino, M. A., Mezzalira, A. and Bertolini, M. (2009). Developmental potential of bovine hand-made clone embryos reconstructed by aggregation or fusion with distinct cytoplasmic volumes. *Cloning and Stem Cells*, **11**(3), 377–386. doi: 10.1089/clo.2009.0022
Ribeiro, E. S., Galvão, K. N., Thatcher, W. W. and Santos, J. E. P. (2012). Economic aspects of applying reproductive technologies to dairy herds. *Animal Reproduction*, **9**, 370–387.
Rizos, D., Ward, F., Duffy, P., Boland, M. P. and Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development*, **61**(2), 234–248. doi: 10.1002/mrd.1153
Rocha-Frigoni, N. A., Leão, B. C., Nogueira, É., Accorsi, M. F. and Mingoti, G. Z. (2014). Reduced levels of intracellular reactive oxygen species and apoptotic status are not correlated with increases in cryotolerance of bovine embryos produced in vitro in the presence of antioxidants. *Reproduction, Fertility, and Development*, **26**(6), 797–805. doi: 10.1071/RD12354
Rodríguez-González, E., López-Bejar, M., Mertens, M. J. and Paramio, M. T. (2003). Effects on in vitro embryo development and intracellular glutathione content of the presence of thiol compounds during maturation of prepubertal goat oocytes. *Molecular Reproduction and Development*, **65**(4), 446–453. doi: 10.1002/mrd.10316
Sakurai, N., Takahashi, K., Emura, N., Fujii, T., Hirayama, H., Kageyama, S., Hashizume, T. and Sawai, K. (2016). The necessity of OCT-4 and CDX2 for early development and gene expression involved in differentiation of inner cell mass and trophectoderm lineages in bovine embryos. *Cell Reprogram*, **18**(5), 309–318. doi: 10.1089/cell.2015.0081
Schreck, R., Rieber, P. and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO Journal*, **10**(8), 2247–2258. doi: 10.1002/j.1460-2075.1991.tb07761.x
Seidel, G. E. (2006). Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology*, **65**(1), 228–235. doi: 10.1016/j.theriogenology.2005.09.025
Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*, **82**(2), 291–295. doi: 10.1113/expphysiol.1997.sp004024
Stevens, R., Stevens, L. and Price, N. C. (1983). The stabilities of various thiol compounds used in protein purifications. *Biochemical Education*, **11**(2), 70. doi: 10.1016/0307-4412(83)90048-1
Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P. B. and Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: Correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. *Biology of Reproduction*, **64**(3), 904–909. doi: 10.1095/biolreprod64.3.904
Stringfellow, D. A. and Givens, M. D. (2010). *Manual of the International Embryo Transfer Society: A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures*, 4th edn. Savory, Ill.: International Embryo Transfer Society.
Sudano, M. J., Paschoal, D. M., Rascado, Tda S., Magalhães, L. C., Crocomo, L. F., de Lima-Neto, J. F. and Landim-Alvarenga, Fda C. (2011) Lipid content and apoptosis of *in vitro*-produced bovine embryos as determinants of susceptibility to vitrification. *Theriogenology*, **75**(7), 1211–1220. doi: 10.1016/j.theriogenology.2010.11.033
Takahashi, M., Nagai, T., Hamano, S., Kuwayama, M., Okamura, N. and Okano, A. (1993). Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryos. *Biology of Reproduction*, **49**(2), 228–232. doi: 10.1095/biolreprod49.2.228
Takahashi, H., Kuwayama, M., Hamano, S., Takahashi, M., Okano, A., Kadokawa, H., Kariya, T. and Nagai, T. (1996). Effect of b-mercaptoethanol on the viability of IVM/IVF/IVC bovine embryos during long-distance transportation in plastic straws. *Theriogenology*, **46**(6), 1009–1015. doi: 10.1016/s0093-691x(96)00265-8
Takeichi, M. (1988). The cadherins: cell–cell adhesion molecules controlling animal morphogenesis. *Development*, **102**(4), 639–655. doi: 10.1242/dev.102.4.639
Takeo, T., Horikoshi, Y., Nakao, S., Sakoh, K., Ishizuka, Y., Tsutsumi, A., Fukumoto, K., Kondo, T., Haruguchi, Y., Takeshita, Y., Nakamuta, Y., Tsuchiyama, S. and Nakagata, N. (2015). Cysteine analogs with a free thiol group promote fertilization by reducing disulfide bonds in the zona pellucida of mice. *Biology of Reproduction*, **92**(4), 90. doi: 10.1095/biolreprod.114.125443
Tarín, J. J., Ten, J., Vendrell, F. J. and Cano, A. (1998). Dithiothreitol prevents age-associated decrease in oocyte/conceptus viability *in vitro*. *Human Reproduction*, **13**(2), 381–386. doi: 10.1093/humrep/13.2.381
Truong, T. and Gardner, D. K. (2017). Antioxidants improve IVF outcome and subsequent embryo development in the mouse. *Human Reproduction*, **32**(12), 2404–2413. doi: 10.1093/humrep/dex330
Truong, T. T. and Gardner, D. K. (2020). Antioxidants increase blastocyst cryosurvival and viability post-vitrification. *Human Reproduction*, **35**(1), 12–23. doi: 10.1093/humrep/dez243
Truong, T. T., Soh, Y. M. and Gardner, D. K. (2016). Antioxidants improve mouse preimplantation embryo development and viability. *Human Reproduction*, **31**(7), 1445–1454. doi: 10.1093/humrep/dew098
Tsuzuki, Y., Saigoh, Y. and Ashizawa, K. (2005). Effects of β-mercaptoethanol on ATP contents in cumulus cell-enclosed bovine oocytes matured in vitro and sequential development of resultant embryos from in vitro fertilization. *Journal of Mammalian Ova Research*, **22**(1), 33–39. doi: 10.1274/jmor.22.33

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press

Vajta, G., Holm, P., Greve, T. and Callesen, H. (1997). The submarine incubation system, a new tool for *in vitro* embryo culture: A technique report. *Theriogenology*, **48**(8), 1379–1385. doi: 10.1016/S0093-691X(97)00379-8

Van Soom, A., Yuan, Y. Q., Peelman, L. J., de Matos, D. G., Dewulf, J., Laevens, H. and de Kruif, A. (2002). Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. *Theriogenology*, **57**(5), 1453–1465. doi: 10.1016/S0093-691X(01)00726-9

Velez-Pardo, C., Morales, A. T., Del Rio, M. J. and Olivera-Angel, M. (2007). Endogenously generated hydrogen peroxide induces apoptosis via mitochondrial damage independent of NF-kappaB and p53 activation in bovine embryos. *Theriogenology*, **67**(7), 1285–1296. doi: 10.1016/j.theriogenology.2007.01.018

Viana, J. (2020). 2019 Statistics of embryo production and transfer in domestic farm animals. *Embryo Technology Newsletter IETS*, **38**, 7–26.

Werlich, D. E., Barreta, M. H., Martins, L. T., Vieira, A. D., Moraes, A. N. and Mezzalira, A. (2006). Bovine IVP embryos vitrified in different cryoprotectant solutions, using or not super cooled nitrogen. *Acta Scientiae Veterinariae*, **34**, 77–82.

Willert, K. and Nusse, R. (1998). Beta-catenin: A key mediator of Wnt signaling. *Current Opinion in Genetics and Development*, **8**(1), 95–102. doi: 10.1016/s0959-437x(98)80068-3

Wong, S., Kirkland, J. L., Schwanz, H. A., Simmons, A. L., Hamilton, J. A., Corkey, B. E. and Guo, W. (2014). Effects of thiol antioxidant β-mercaptoethanol on diet-induced obese mice. *Life Sciences*, **107**(1–2), 32–41. doi: 10.1016/j.lfs.2014.04.031

https://doi.org/10.1017/S0967199422000338 Published online by Cambridge University Press