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Comparison of CellRox green fluorescence upon thawing on *in vitro Bos taurus* and *Bos indicus* embryos cryopreserved by slow freezing or vitrification

David Osornio[1], David Alejandro Contreras[1], Edgar Jimenez-Diaz[2], Tatiana Fiordelisio[2,3], Patricia López-Damian[3], José Francisco Martínez[4] and Carlos Salvador Galina[4]

[1]Departamento de Medicina y Zootecnia de Rumiantes, Facultad de Medicina Veterinaria y Zootecnia; [2]Unidad de Imagenologia Cuantitativa, Laboratorio Nacional de Soluciones Biomiméticas para Diagnóstico y Terapia LaNSBioDyT; [3]Laboratorio de Neuroendocrinología Comparada, Departamento de Ecología y Recursos Naturales, Biología, Facultad de Ciencias and [4]Departamento de Reproducción, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Mexico City, Mexico

Research Article

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Corresponding author:
Tatiana Fiordelisio;
Email: tfiorde@ciencias.unam.mx

Summary

The aim of this study was to compare the levels of reactive oxygen species (ROS) in *Bos taurus* and *Bos indicus in vitro* embryos cryopreserved using either slow freezing or vitrification. Embryos were divided into four groups based on subspecies and freezing method: *Bos indicus* slow freezing (BiSF; $n = 8$), *Bos indicus* vitrification (BiVT; $n = 10$), *Bos taurus* slow freezing (BtSF; $n = 9$), and *Bos taurus* vitrification (BtVT; $n = 6$). After thawing, the embryos were incubated with CellRox Green and images were obtained using a confocal microscope. The fluorescence intensity of each cell was measured and expressed as arbitrary units of fluorescence (auf) and compared using a multiple regression and unpaired *t*-test with $\alpha = 0.05$. Results showed that subspecies and the freezing method significantly affected auf ($P < 0.001$; $R^2 = 0.1213$). *Bos indicus* embryos had higher auf than *Bos taurus* embryos, whether frozen by slow freezing (67.05 ± 23.18 vs 51.30 ± 16.84, $P < 0.001$) or vitrification (64.44 ± 23.32 vs 47.86 ± 17.53, $P < 0.001$). Slow freezing induced higher auf than vitrification in both *Bos taurus* (51.30 ± 16.84 vs 47.86 ± 17.53, $P < 0.001$) and *Bos indicus* (67.05 ± 23.18 vs 64.44 ± 23.32, $P < 0.014$). In conclusion, *Bos taurus* embryos had lower ROS levels when frozen using vitrification, while *Bos indicus* embryos had consistent ROS patterns regardless of the freezing method. However, *Bos indicus* embryos frozen by slow freezing tended to have a higher number of cells with elevated ROS levels.

Introduction

In the past, the industrial production of embryos comprised a cumbersome and expensive protocol based on superovulation and uterine flushing (Lamb *et al.*, 2016). Therefore, *in vitro* embryo production has become the most popular technique (Viana *et al.*, 2018). Nonetheless, bovine embryos produced *in vitro* still differ from those produced *in vivo* in terms of morphology, total cell numbers, and sensitivity to cold shock (Mucci *et al.*, 2006). In addition, metabolic differences leading to decreased ability to survive cryopreservation have been reported (Stinshoff *et al.*, 2011).

There are two commonly used methods to cryopreserve bovine embryos: slow freezing (SF) and vitrification (VT). In general, the freeze–thaw process in either procedure generates reactive oxygen species (ROS) through metabolic and biochemical changes occurring during hypoxia and re-oxygenation (Chatterjee and Gagnon, 2001). Hypothermia-hypoxia generated by freezing reduces ATP levels (Hochachka, 1986), along with the uncoupling of the inner mitochondrial membrane, causing an increase in oxygen levels (McCord, 2000). Following thawing, re-oxygenation facilitates the formation of free radicals and the decrease in antioxidant activity, leading to a state of oxidative stress (Agarwal *et al.*, 2006; Tatone *et al.*, 2010).

Even though it is inevitable for the embryos to fall into a state of oxidative stress from cryopreservation and thawing, it has been well documented that *Bos taurus* embryos after thawing, display higher cryotolerance when produced *in vivo* than *in vitro* (Sudano *et al.*, 2014 and when frozen by VT compared with SF (Do *et al.*, 2019). In fact, Walton *et al.* (2017) showed with their studies that vitrification of bovine embryos is better than SF as it ensures a higher percentage of embryo development when comparing survival rates between SF versus vitrification during expansion and hatching. Arshad *et al.* (2021) in their meta-analysis comparing SF versus VT involving *in vitro* and *in vivo Bos taurus*-derived embryos, concluded

that, even though VT displays higher survival rates and short-term protective effects against cryodamage, this might dysregulate genes related to pregnancy success post-embryo transfer. Furthermore, pregnancy rates post-transfer showed similarities between frozen by SF and VT (Arshad *et al.*, 2021).

For *Bos indicus* embryos, a higher content of lipid droplets compared with those derived from *Bos taurus* may lead to their fragmentation and release into the cytoplasm, causing cellular toxicity, triggering oxidative stress, and probably leading to lower embryo viability (Contreras *et al.*, 2021). In fact, our previous research has shown that *Bos indicus* bovine embryos produced *in vivo* displayed higher amounts of lipid droplets (López-Damián *et al.*, 2018) and ROS (López-Damián *et al.*, 2020) compared with *Bos taurus*, both fresh and after SF. Therefore, it was of interest to verify whether this discrepancy between the two subspecies was maintained in frozen embryos produced *in vitro* or whether VT could reduce the amount of ROS after thawing.

Materials and methods

Ethical statement

The Comité Institucional para el Cuidado y Uso de Animales de Experimentación of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Nacional Autónoma de México approved the methods used during the present work in accordance with the International Code of Medical Ethics of the World Medical Association (Declaration of Helsinki).

Embryo production

Bovine embryos were produced *in vitro* at the Centro de Desarrollo de Capacidad Productiva y Mejoramiento Genético de la Ganadería at the Unión Ganadera Regional de Tamaulipas (UGRT) and frozen during the years 2020 and 2021. The ovaries from Charolais and Brahman breeds were collected from a local abattoir and transported to the laboratory in a saline solution (0.9% NaCl) at a temperature of 30°C, supplemented with 100 IU/ml of penicillin and 50 µg/ml of streptomycin sulfate.

The cumulus–oocyte complexes (COCs) were obtained from follicles ranging from 3 to 8 mm in diameter and washed with HEPES 1 M (Gibco Labs, Grand Island, NY, USA) and evaluated under a stereomicroscope. The COCs were cultured for 24 h in an incubator at 38.5°C in a saturated humidity atmosphere containing 5% CO_2 and 95% air. The maturation medium utilized consisted of TCM199 containing Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA), with the addition of FSH (100 ng/ml; Follitropin-V®, Vetoquinol, CDMX, Mexico) 25-mM HEPES 1M, 0.2-mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 0.4% fatty acid-free BSA, 100 IU/ml penicillin, and 50 µg/ml streptomycin sulfate.

After the culture period, the COCs were evaluated for cumulus expansion using a subjective grading system: a score of 1 indicated poor expansion, characterized by minimal morphological changes compared with the pre-maturation state; a score of 2 indicated partial expansion, with reasonable expansion observed in some but not all COCs; and a score of 3 indicated complete or nearly complete expansion of all COCs (Kobayashi *et al.*, 1994). Immediately after the assessment of cumulus expansion, COCs were denuded by 3 min of vortexing.

The matured oocytes were placed into Fert-TALP medium and inseminated with a tested frozen semen pool from two sires (Charolais and Brahman). The semen was separated with 90% Percoll and diluted to a final concentration of 2×10^6 sperm/ml in Fert-TALP medium containing 10 µg/ml heparin, 20 µg/ml penicillamine, 10 µM hypotaurine, and 1 µM epinephrine. *In vitro* fertilization was performed by co-culture of sperm and oocytes for 18 h in 4-well plates under the same atmospheric conditions as the ones used for maturation. After gamete co-incubation, the cumulus cells were removed with a vortex for a 2-min period.

Presumptive zygotes were cultured at 38.5°C in 400 µl synthetic oviduct fluid (SOFaaci; Holm *et al.*, 1999) medium in 4-well plates (Nunc, Roskilde, Denmark) under a saturated humidity atmosphere containing 5% CO_2, 5% O_2, and 90% N_2 until the embryos were at the blastocyst stage and classified as quality category 1 (Bó and Mapletoft, 2018).

Freezing

The final breeds of the embryos used for this study were pure Charolais of the subspecies *Bos taurus* and pure Brahman of the subspecies *Bos indicus*. Embryos were cryopreserved either by SF or vitrification. Embryos were organized into four groups according to subspecies and freezing method: (1) *Bos indicus* SF (BiSF; $n = 8$), (2) *Bos indicus* vitrification (BiVT; $n = 10$), (3) *Bos taurus* SF (BtSF; $n = 9$), and (4) *Bos taurus* vitrification (BtVT; $n = 6$). Each group had a positive sample and a negative sample that served as quality controls.

Vitrification

A 60-µl droplet of Wash medium (Vitrogen, São Paulo, Brazil) followed by two 45-µl droplets of V1 and two additional 45-µl droplets of V2 (Vitrogen, São Paulo, Brazil) were used. Subsequently, the embryos from the dish, initially in the *in vitro* cultured medium, were moved to the Wash medium. Then, embryos were transferred to the first V1 droplet for a duration of 1 min, followed by the second V1 droplet for 7 min. After this, they were immersed in the first V2 droplet for 20 s and then transferred to the second V2 droplet. After ~20 s of immersion, embryos were loaded into the Cryotop®, which was subsequently submerged in liquid nitrogen.

Slow freezing

For this procedure, a modified approach that involved V1, combined with a solution of ethylene glycol and sucrose was used. The necessary medium, placing three 60-µl droplets of Wash medium, alongside a single 45-µl droplet in a Petri dish. In another Petri dish, ethylene glycol was maintained at a temperature of 38.5° C. Subsequently, embryos were transferred from the dish with *in vitro* cultured medium to the first Wash medium drop, and in the other two drops. Once in the third drop, they were transferred to V1 for a duration of 2 min and then immersed in ethylene glycol for 4 min. During this 4-min period, they were pipetted into straws. After the specified time, the straws containing the embryos were placed in a controlled embryo freezer CRYSALYS® PTC-9500 device, which managed the automated temperature reduction process. Subsequently, these were exposed to a 2-min period for stabilization at a temperature of −7°C. Following this interval, the straws were filled, followed by an 8-min stabilization at −7°C. Finally, the temperature was gradually lowered at a rate of 0.5°C per min until it reached −35°C within 60 min, at which point the straws were submerged in liquid nitrogen for cryopreservation.

Thawing and rehydration

Embryo straws were thawed by keeping them for 5 s at room temperature and then immersed in a water bath at 30°C for 30 s.

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Rehydration was carried out for 10 min, starting by transferring them from cryoprotectant to 200 ml of holding medium (Holding Vigro Plus, Vetoquinol, Canada) at 30°C for 4 min. Then, they were incubated (Labnet International, Inc., Edison NJ, USA) in two medium holding changes with 300 ml and 400 ml for 4 min and 2 min, respectively, gradually increasing the temperature until reaching 37°C.

Determination of reactive oxygen species

The embryos, after thawing, were incubated at 37°C with a pro-fluorescent molecule (CellRox Green, Invitrogen Molecular Probes, USA) that, when oxidized by ROS, gave a detectable fluorescence signal to measure the quantity of ROS present in the embryos. All embryos were placed in 200 ml of 5 µM CellRox Green in holding medium and incubated at 37°C for 30 min while being shielded from the light (Labnet International, Inc., Edison NJ, USA). After this, embryos were washed twice with holding medium at 37°C before being fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature. Then, the nuclei were stained with DAPI (Invitrogen, Molecular Probes, USA) for 15 min, followed by three rinses with D-PBS (Sigma-Aldrich, St. Louis, MO, USA) containing polyvinylpyrrolidone (PVP; Sigma-Aldrich, St. Louis, MO, USA) as a surfactant (1 mg/ml). The embryos were then mounted between coverslips that had been pre-prepared with 30% poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) using 15 ml of Dako mounting medium and then was rinsed three times with D-PBS/PVP (Dako North America Inc, Carpinteria, CA, USA).

Four embryos of each experimental group were used as negative controls as these cells show high levels of autofluorescence (Gardner *et al*., 2004). The procedure followed was the same as for the experimental embryos, with the absence of CellRox Green. As quality controls, four embryos were handled for each experimental group using the same methods described for the experimental embryos, with the addition of CellRox Green after a pre-incubation (37°C, 30 min) in 100 µM of cumene hydroxide as an oxidizing agent.

Imaging acquisition

The images were acquired during the first 24 h after being cultured in CellRox Green, and the parameters were set based on the positive control of the subspecies that recorded the highest fluorescence intensity, and were uniform for all embryos used in each procedure. The images were captured using a dry HC PL Fluotar ×20/0.50 objective in a confocal microscope (Leica TCS SP8, Germany). The 488 nm diode laser line with an excitation/emission of 485/520 nm was utilized to detect ROS, and the 405 nm laser line with a 359/461 nm excitation/emission for DAPI. Each channel was captured independently, and then the photographs were combined and saved in TIF format using the Leica Application Suite X (LAS X) software.

Image analysis

The Fiji program (https://fiji.sc/) was used to quantify the fluorescence intensity (mean grey value). The RGB image was divided and saved as 8 bits. The Region of Interest (ROI) was used to delimit each cell, and the mean grey value was calculated. The average intensity of four background ROIs was measured and subtracted from each cell value to obtain the adjusted mean intensity of fluorescence, which was then used to correct the fluorescence data. Additionally, the average baseline fluorescence assessed in the negative controls was subtracted from each measurement to reduce the contribution of autofluorescence present in the embryos. The CellRox Green fluorescence calculated for each cell was then expressed in arbitrary units of fluorescence (auf).

Statistical analysis

Data were analyzed using a least squares multiple regression model assuming a normal distribution of the residuals. The auf per cell area was chosen as the dependent variable (Y) with the main effects (X1 and X2) being subspecies (two categorical levels; *Bos indicus* = 1, and *Bos taurus* = 0) and freezing method (two categorical levels; SF = 1, and vitrification = 0). Then an unpaired *t*-test with Welch correction was performed to compare CellRox Green auf between subspecies and freezing method, and were expressed as mean ± standard deviation. All models were assessed at a significance level of $\alpha = 0.05$ using the software GraphPad Prism version 9.

Results

As seen in Table 1, the parameters, subspecies, and freezing method were significant in modelling their effects on CellRox Green auf ($P < 0.001$; R2 = 0.1213). Comparing each cell auf across the image Z-axis, *Bos indicus* embryos showed higher CellRox Green auf than *Bos taurus*, both by SF (67.05 ± 23.18 vs. 51.30 ± 16.84, $P < 0.001$) and vitrification (64.44 ± 23.32 vs. 47.86 ± 17.53, $P < 0.001$) (Figure 1). The SF method had a higher CellRox Green auf when compared with vitrification, both in *B. taurus* (51.30 ± 16.84 vs. 47.86 ± 17.53, $P < 0.001$) and *B. indicus* (67.05 ± 23.18 vs. 64.44 ± 23.32, $P < 0.014$) (Figure 2).

In Figure 1, when comparing the distribution of cells between BiSF vs BtSF (top panel), the proportion of cells with auf above their respective means was higher in BiSF (mean 67.05; 44.30%) than in BtSF (mean 51.30; 40.38%). The cell distribution displayed a higher range of auf below the Q1 in BiSF (Q0: 20.63; Q1: 50.27) than in BtSF (Q0: 21.39; Q1: 38.17). Additionally, the cell distribution displayed a higher range of auf above the Q3 in BiSF (Q3: 84.68; Q4: 120.71) than in BtSF (Q3: 65.85; Q4: 91.14). Similarly, when comparing the distribution of cells between BiVT vs BtVT (bottom panel), the proportion of cells with auf above their respective means was higher in BiVT (mean 64.44; 49.87%) than in BtVT (mean 47.86; 42.83%). The cell distribution displayed a higher range of auf below the Q1 in BiVT (Q0: 19.10; Q1: 45.83) than in BtVT (Q0: 19.04; 33.85). Furthermore, the cell distribution displayed a higher range of auf above the Q3 in BiVT (Q3: 83.50; Q4: 119.72) than in BtVT (Q3: 61.34; Q4: 90.35).

In Figure 2, when comparing the distribution of cells between BiSF and BiVT (top panel), the proportion of cells with auf above their respective means was lower in BiSF (mean 67.05; 44.30%) than in BiVT (mean 64.44; 49.87%). The cell distribution displayed a higher range of auf below Q1 in BiSF (Q0: 20.63; Q1: 50.27) than in BiVT (Q0: 19.10; Q1: 45.83). Additionally, the cell distribution displayed a higher range of auf above Q3 in BiSF (Q3: 84.68; Q4: 120.71) than in BiVT (Q3: 83.50; Q4: 119.72). When comparing the distribution of cells between BtSF and BtVT (bottom panel), the proportion of cells with auf above their

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Table 1. Least square multiple regression modelling of CellRox Green auf for freezing method and bovine subspecies

Variable	Estimate	Standard error	95% CI (asymptotic)	|*t*|	*P*-value
Intercept	48.16	0.7326	46.73–49.60	65.75	<0.001
Subspecies (*Bos indicus)*	16.13	0.759	14.64–17.62	21.25	<0.001
Freezing method (slow freezing)	2.937	0.7439	1.479–4.396	3.949	<0.001

Categorical levels within the parentheses were used to compute the estimates, with a value of 1 assigned to each level.

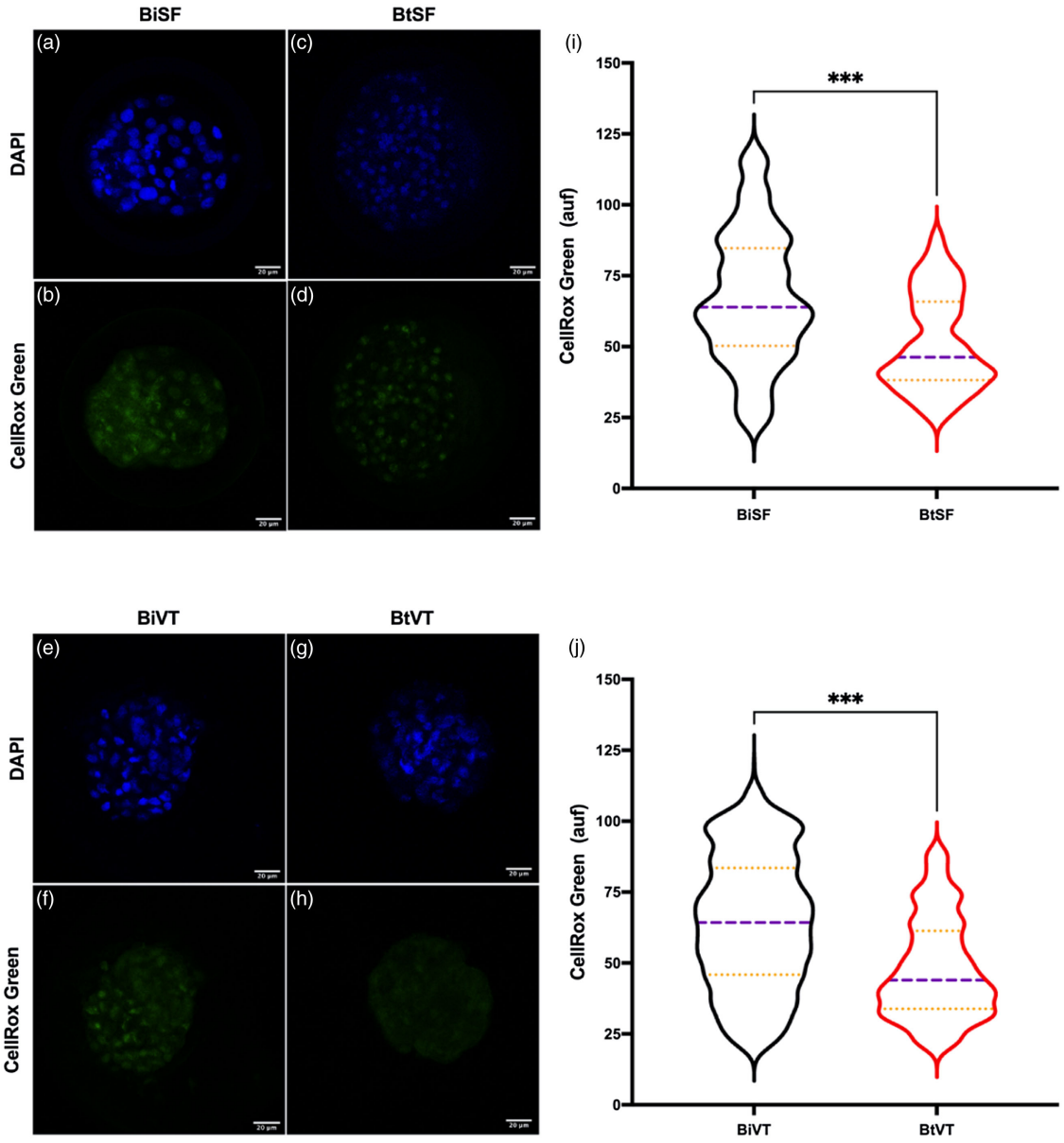

Figure 1. Comparison of CellRox Green auf between *Bos indicus* and *Bos taurus* embryos frozen by slow freezing and vitrification after thawing. Top panel: *Bos indicus* slow freezing (BiSF; $n = 860$) versus *Bos taurus* slow freezing (BtSF; $n = 790$). Bottom panel: *Bos indicus* vitrification (BiVT; $n = 1103$) versus *Bos taurus* vitrification (BtVT; $n = 523$). (a–h) Fluorescence images of quality 1 blastocyst. Green shows the oxidation of a ROS-sensitive sensor (CellRox Green) and blue shows nuclei (DAPI). (i, j) Violin plots of CellRox Green auf (arbitrary units of fluorescence) per cell showing the frequency (width), Q1 and Q3 (orange dotted line) and mean (purple dotted dash line). Unpaired *t*-test with Welch correction, significant differences are expressed as $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.002$ (**), $P < 0.001$ (***). Scale bars 20 µm.

respective means was lower in BtSF (mean 51.30; 40.38%) than in BtVT (mean 47.86; 42.83%). The cell distribution displayed a higher range of auf below Q1 in BtSF (Q0: 21.39; Q1: 38.17) than in BtVT (Q0: 19.04; Q1: 33.85). Additionally, the cell distribution displayed a lower range of auf above Q3 in BtSF (Q3: 65.85; Q4: 91.14) than in BtVT (Q3: 61.34; Q4: 90.35).

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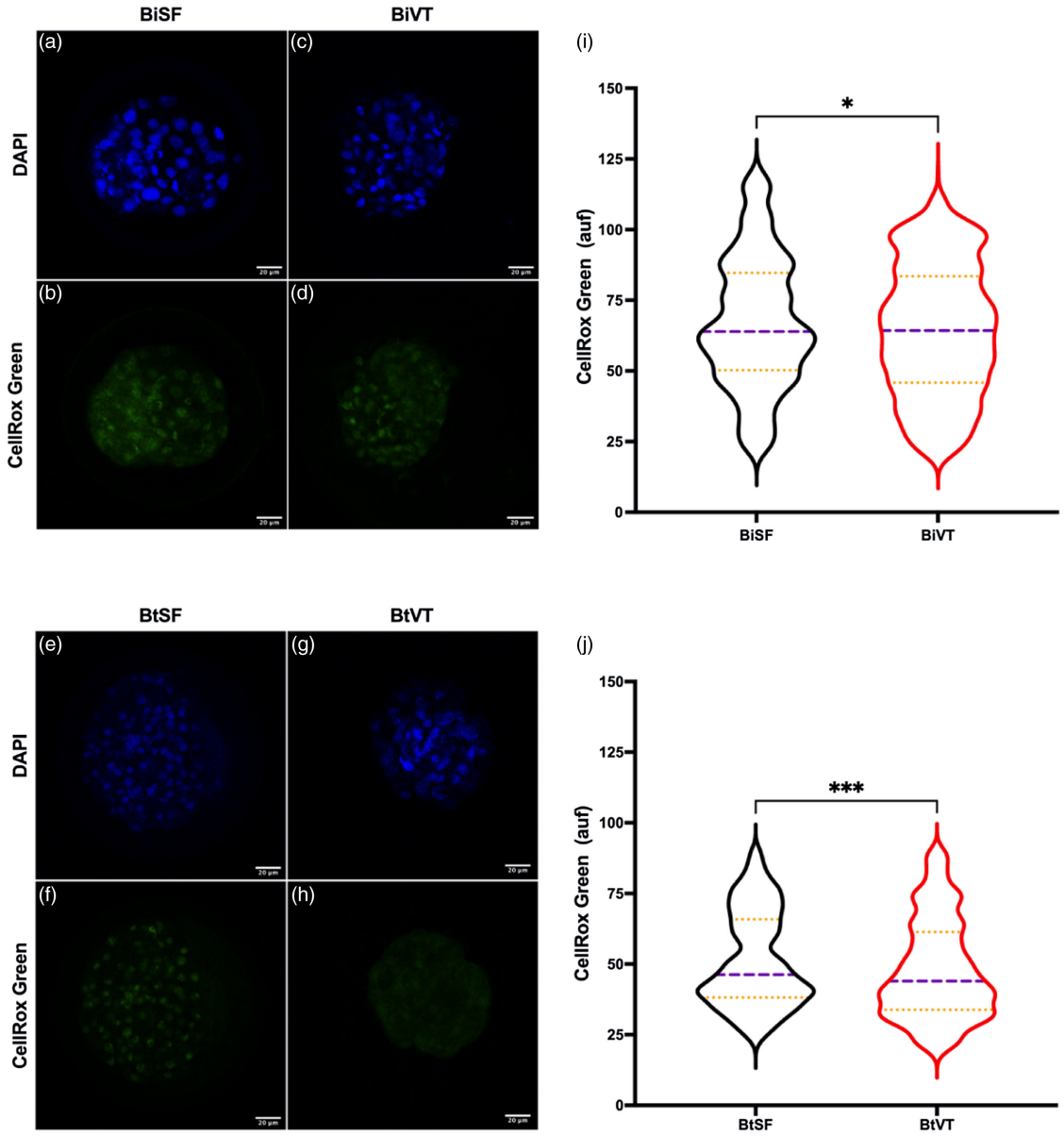

Figure 2. Comparison of CellRox Green auf between *Bos indicus* and *Bos taurus* embryos frozen by slow freezing and vitrification after thawing. Top panel: *Bos indicus* slow freezing (BiSF; $n = 860$) versus *Bos indicus* vitrification (BiVT; $n = 1103$). Bottom panel: *Bos taurus* slow freezing (BtSF; $n = 790$) versus *Bos taurus* vitrification (BtVT; $n = 523$). (a–h) Fluorescence images of quality 1 blastocysts. Green indicates the oxidation of a ROS-sensitive sensor (CellRox Green), while blue indicates nuclei (DAPI). (i, j) Violin plots of CellRox Green auf (arbitrary units of fluorescence) per cell showing the frequency (width), Q1 and Q3 (orange dotted line), and mean (purple dotted dash line). Unpaired *t*-test with Welch correction, significant differences are expressed as $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.002$ (**), $P < 0.001$ (***). Scale bars 20 µm.

Discussion

Table 1 summarizes the effects of multiple linear regression when modelling the variables subspecies and freezing method on the ROS fluorescence peculiarities in the inner cell mass of the embryos. As can be seen, there is an increase in fluorescence, either in the subspecies or freezing method, when considering the data on *Bos taurus* and vitrification as the baseline, particularly when comparing these data with *Bos indicus*. These figures are in accordance with previous research undertaken to evaluate the morphological characteristics of *Bos indicus* embryos (Visintin *et al.*, 2002; López-Damián *et al.*, 2018). As expected, there were differences between *Bos taurus* and *Bos indicus*, as the latter displayed higher levels of ROS. In fact, López-Damián *et al.* (2020) found that fresh embryos cryopreserved via SF and exposed to the CellRox Green procedure showed higher levels of ROS in *Bos indicus* embryos than in *Bos taurus*, both fresh and after freezing. Considering that a higher accumulation of lipid droplets in the cytoplasm of *in vitro*-derived embryos, was observed, compared with those *in vivo*, this might be the result of differences in the lipid content from animal-derived components in the culture medium, such as fetal calf serum (McEvoy *et al.*, 2001), or mitochondrial dysfunction to metabolize lipids under *in vitro* conditions (Dorland, 1994), affecting ROS production. Our results, using *in vitro*-derived embryos, tended to confirm that this trend could be similar when exposed to the freezing methods used in the present study. For *Bos taurus* embryos, freezing capabilities were similar to those previously reported (López-Damián *et al.*, 2020).

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When comparing different freezing methods for *in vitro*-produced embryos, our results showed a higher amount of ROS in SF compared with VF. Arshad *et al.* (2021), in their meta-analysis, demonstrated that vitrification tended to produce fewer levels of ROS when compared with SF. They claim that this disparity could be explained by the response of the embryos to vitrification, which has been shown to reduce the uptake of cholesterol and fatty acids from culture medium, resulting in a lesser accumulation of cytoplasmic lipids (Gupta *et al.*, 2017).

Although vitrification appears to be a better technique for freezing embryos, our results indicated that, at least in *Bos indicus*, there were no major differences in relation to the chosen freezing method. However, for *Bos indicus* embryos, vitrification reduced the number of cells displaying higher ROS levels. How do these methods affect fertility? The vitrification technique has been shown to increase embryo survivability when compared with SF in *in vitro*-produced embryos (Walton *et al.*, 2017). Also, Zárate-Guevara *et al.* (2018), evaluating the post-transfer percentage of pregnancy with *in vivo Bos indicus* × *Bos taurus* embryos cryopreserved by either SF or vitrification, found a higher percentage of achieved pregnancy in cows transferred with embryos frozen by vitrification (64%) than with SF (8%). Nonetheless, Arshad *et al.* (2021), with data derived from *Bos taurus*, found similarities in the risk of becoming pregnant by either SF or vitrification. More research comparing fertility data should be encouraged to discern how different were these two subspecies of embryos.

The differences observed in *Bos indicus* embryos tend to confirm our previous data (Godinez *et al.*, 2013; López-Damián *et al.*, 2020; Marquez *et al.*, 2005) that there are important morphological and adaptative differences between *Bos taurus* embryos as opposed to *Bos indicus*. In fact, López-Damián *et al.* (2018) showed marked disparities in the array of lipid droplets in the cytoplasm of the former, suggesting difficulties in the survival of the embryos at thawing, particularly those not accurately classified (Aguilar *et al.*, 2002). In conclusion, *Bos taurus* embryos had lower ROS levels when frozen using vitrification, while *Bos indicus* embryos had consistent ROS patterns regardless of the freezing method. However, *Bos indicus* embryos frozen by SF tended to have more cells with high ROS levels.

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