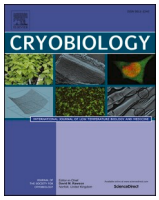
Cryobiology 117 (2024) 104981

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo



Impact of lipid content on oxygen reactive species and viability predictors in vitrified bovine embryos

Clara Slade Oliveira [a,*], Viviane Luzia da Silva Feuchard [a,b], Carolina Romano Capobiango Quintão [a], Leticia Zoccolaro Oliveira [b], Naiara Zoccal Saraiva [a]

[a] Embrapa Dairy Cattle, Eugênio do Nascimento Ave. 610, 36038-330, Juiz de Fora, MG, Brazil
[b] Universidade Federal de Minas Gerais, Department of Veterinary Clinics and Surgery, Veterinary School, Presidente Antônio Carlos Ave., 31270-901, Belo Horizonte, MG, Brazil

ARTICLE INFO

Keywords:
Vitrification
Lipid content
Oxidative stress
Embryo
Cryotolerance

ABSTRACT

Given the significant variation in lipid levels among bovine embryos, our study was designed to associate lipid content to oxidative stress in individual embryos undergoing vitrification, and to assess how this and other morphological parameters impacts cryosurvival. Linear and logistic regression were performed to understand the influence of the variables in the cryosurvival. T-test or Kruskal Wallis were employed to compare means. Vitrified embryos revealed a positive correlation between lipid content and oxidative stress post-warming both 2 h ($p = 0.025$, $n = 64$) and 48 h ($p < 0.001$, $n = 122$) after warming. Lipid levels explained ($p < 0.001$) up to 51 % (multiple R-squared) of oxidative stress variability. Compared to fresh embryos, a negative influence ($p = 0.01$) of vitrification-warming procedures was detected in lipid levels. Vitrified embryos exhibited lower ($p < 0.001$, $n = 90$) mean lipid content compared to fresh counterparts 48 h post-warming, and similar ($p = 0.24$) oxidative stress levels. No impact of lipid content or oxidative stress levels was detected on hatchability or embryo quality 48 h post-warming ($n = 99$). Expansion just after (0 h) and 2 h after warming resulted in a higher chance of hatching ($p = 0.015$ and $p = 0.008$, OR 1.30 and 1.58), and a positive association was observed between expansion at 0 h ($p = 0.002$) and embryo area ($p = 0.047$) with cell number. In conclusion, a decrease in lipid levels was found following vitrification-warming procedure and an individual association between lipids and oxidative stress is present in vitrified embryos. Lipids or oxidative stress levels was not linked to survivability of vitrified embryos 48 h following warming. Expansion at 0 h indicates a better chance for hatching and higher cell numbers in vitrified embryos.

1. Introduction

In bovine assisted reproductive technologies (ART), embryo cryopreservation is crucial for genetic preservation and reproductive planning. However, achieving optimal cryotolerance in in vitro produced (IVP) embryos is notably more challenging than with in vivo embryos [6]. A key factor contributing to this difference is the lipid content within the cytoplasm, which has garnered considerable attention due to its intricate relationship with embryo viability [15]. The lipid profile plays a role in cryotolerance; for instance, conjugated linoleic acid supplementation can alter the composition of structural lipids in cellular membranes, increasing levels of highly unsaturated phosphatidylcholines and enhancing cryosurvival [11]. Despite the significance of lipid profile, this study will specifically focus on the quantity of neutral lipids in the cytoplasm.

Excessive lipid droplet formation negatively impacts cryotolerance [23,24]. Fatty acids in the culture medium can disrupt mitochondrial function and lipid metabolism, leading to a distinct molecular profile in embryos characterized by higher levels of reactive oxygen species (ROS), mitochondrial dysfunction, and lipid accumulation in bovine embryos [20]. Despite efforts to mitigate lipid accumulation such as serum reduction and supplementation with delipidating agents, achievement of optimal cryotolerance remains elusive. Interestingly, while in vitro produced embryos typically have higher lipid content compared to in vivo embryos, reducing lipid levels does not always correlate with improved cryotolerance [26]. This suggests that lipid

* Corresponding author. Animal Reproduction Laboratory, Embrapa Dairy Cattle, Juiz de Fora, Brazil.
E-mail addresses: clara.oliveira@embrapa.br (C.S. Oliveira), viviane.lfeuchard22@gmail.com (V.L.S. Feuchard), carolina.quintao@embrapa.br (C.R.C. Quintão), leticiazoccolaro@yahoo.com.br (L.Z. Oliveira), naiara.saraiva@embrapa.br (N.Z. Saraiva).

https://doi.org/10.1016/j.cryobiol.2024.104981
Received 28 June 2024; Received in revised form 3 September 2024; Accepted 14 October 2024
Available online 18 October 2024

content alone is not the primary determinant of embryo viability after cryopreservation; rather, the oxidative stress associated with lipid accumulation may reduce cryotolerance [7].

Thus, there is a complex interplay between lipid metabolism and cryotolerance, with oxidative stress emerging as a significant player. Characterized by an imbalance between ROS production and cellular antioxidant defenses, oxidative stress has detrimental effects on embryonic development [4]. Elevated ROS levels during in vitro culture, especially in high oxygen tension environments, lead to metabolic alterations, mitochondrial dysfunction, and lipid peroxidation, ultimately compromising embryo quality and cryotolerance. Since ROS can damage mitochondrial integrity and activity through lipid peroxidation [9], efforts to reduce oxidative stress with exogenous antioxidants have shown promise in reducing ROS levels and improving embryo cryosurvival after vitrification [14]. Notably, embryos cultured under low oxygen tension show improved metabolic activity and cryotolerance, likely due to enhanced expression of genes involved in lipid metabolism and cholesterol synthesis [13].

Understanding the intricate relationship between lipid content, oxidative stress, and cryotolerance is imperative for optimizing assisted reproductive technology outcomes. Due to the high variability in oxidative stress and lipid content among individual embryos (e.g., in this study, the coefficient of variation was 38 % for oxidative stress levels and 45 % for lipid content among embryos from the vitrified group), grasping the true extent of the relationship between lipid content and cryosurvival is challenging. This challenge arises from the common practice of averaging within a group of embryos, which constrains the ability to link viability to each embryo individually. Therefore, in this study we investigated the associations between lipid content, reactive oxygen species (ROS), and cryosurvival in individual embryos.

2. Material and methods

All experimental procedures followed ethical guidelines for experimentation, in compliance with relevant Brazilian laws and Embrapa guidelines. No live vertebrates were used in this study.

2.1. Experimental design

The study was divided into three main assays. No selection based on lipid amount in embryos was performed – day 7 grade I expanded blastocysts were randomly assigned to either the fresh or vitrified groups.

Assay one aimed to assess the relationship between lipid content and oxidative stress after warming in vitrified embryos. Vitrified embryos were warmed, cultured, stained with CellRox, fixed, stained with Nile Red, and imaged for analysis. Two independent experiments were conducted: 2 h after warming (64 blastocysts from 4 replicates were analyzed; 3 blastocysts that did not expand were excluded) and another at 48 h after warming (122 blastocysts from 5 replicates were analyzed; 15 blastocysts did not expand were excluded).

Assay two was designed to compare fresh and vitrified embryos in terms of lipid content and its relation to oxidative stress. Day 7 blastocysts from the same batch were divided into control (fresh) and vitrified groups (90 blastocysts, 41–49 per group, from 3 replicates were analyzed; 3 blastocysts from the vitrified group that did not expand were excluded). In the vitrified group, embryos were vitrified and warmed immediately after vitrification. Both groups were cultured for up to 48 h, then stained with CellRox, fixed, stained with Nile Red, and imaged for analysis. Embryos were assessed for lipid content, area, oxidative stress, and nuclei area.

Assay three was designed to correlate vitrified embryo viability (hatchability and cell number at day 9) with lipid content, oxidative stress, and other potential predictors (early expansion, zona pellucida thickness, embryo area, debris, and extruded cells) after the vitrification-warming procedure. For this assay, embryos were individually monitored from warming (0 h, equivalent to day 7 in fresh embryos) until the end of the culture (48 h, equivalent to day 9 in fresh embryos). A total of 99 blastocysts were evaluated (76 vitrified-warmed and 23 fresh, from 2 replicates; 7 blastocysts lost ZP during vitrification-warming step and were excluded from hatching or zona pellucida thickness evaluations. Additionally, 4 blastocysts that did not expand in the vitrified group and 11 embryos that arrested at 48 h (7 in the vitrified group and 4 in fresh group) were excluded from staining analysis. Snapshots were taken at 0, 2, 4, 18, 19.5, 21, 22.5, 24, 25.5, 27, and 48 h. At the end of the 48-h culture, embryos were individually stained with CellRox, fixed, and stained for Nile Red and HOECHST. Each embryo was fully traceable from 0 to 48 h, including staining images. Expansion was assessed immediately after, 2 h after and 4 h after warming to understand the importance of early expansion for cryosurvival. Zona pellucida thickness and embryo area were assessed 4 h after warming, aligning with the first evaluation for fresh embryos.

2.2. Oocyte recovery and embryo production

In vitro embryo production was performed as previously described [18]. Briefly, oocytes were obtained from slaughterhouse ovaries. Follicles with less than 8 mm were manually punctured with a 19G needle and syringe. Follicular fluid was transferred to 100 mm plastic dishes and *cumulus* oocyte complexes (COCs) were selected based on a compact nonatretic cumulus with at least one layer of cells and homogeneous cytoplasm or cytoplasm with minor granules. COCs were transferred to 100 μl drops of TCM 199 (Gibco Life Technologies, Grand Island, EUA) supplemented with 10 % FCS (Vitrocell, Campinas, Brazil), 50 UI/mL hCG (Sigma C-1063, Sigma Chemical, St. Louis, MO), 0.5 μg/mL FSH (Folltropin, Vetrepharm, Belleville, ON, Canada), 1 μg/mL estradiol-17β (Sigma E−8875), 2.2 μg/mL sodium pyruvate (Sigma P-4562) and 70 μg/mL amikacin (Sigma A-2324) covered with mineral oil (Sigma M − 8410) for 24 h for maturation. *In vitro* fertilization was performed using frozen straws of semen from a previously tested Nelore bull. Semen was prepared using Percoll 45/90 gradient and co-incubation of oocytes with spermatozoa was performed in TALP medium supplemented with 10 μg/mL heparin and 10ul/mL PHE for 18 h. For embryo culture, presumptive zygotes were submitted to vigorous pipetting and distributed in groups of maximum 20 into 100 μL drops of SOF-AA based medium, which was half replaced at 48 and 120 h.p.i. All steps were performed at 38.5 °C, 5 % CO_2 in atmospheric air and maximum humidity.

2.3. Vitrification-warming procedure

Vitrification and warming were performed as previously described [17]. Briefly, Day 7 (168 h.p.i.) blastocysts and expanded blastocysts were vitrified using conventional two-step protocol. Vitrification was performed in groups of five embryos and stored in liquid nitrogen. TCM-199 buffered with HEPES (Gibco BRL, Grand Island, NY) supplemented with 1.0 mM sodium pyruvate (Gibco BRL, Grand Island, NY), 100UI penicillin and 0.1 mg/ml streptomycin was used as base in all steps. For vitrification, medium was supplemented with 10 % fetal calf serum. Selected embryos were transferred to 7.5 % DMSO +7.5 % ethylene glycol media for 3 min. Next, embryos were washed in 16 % DMSO +16 % ethylene glycol +0.5 M sucrose media and placed in an open vitrification device (Vitrification “fork”, WTA, Cravinhos, Brazil) in a 0.5 μl droplet. After 30 s device was immersed in liquid nitrogen. For warming, an Eppendorf filled with 1 mL of 0.15 M sucrose +10%FCS media was prepared, and vitrification device was placed directly from liquid nitrogen into the top of the Eppendorf, which was kept in vertical position, allowing the embryos to settle in the bottom of the tube. Embryos remained for 6 min in solution. After, embryos were collected from the bottom of the Eppendorf tube and washed in 5 mL 1 % FCS PBS for sucrose removal. Washed embryos were transferred to 50 μl SOF drops in groups of 10 embryos (assays 1 and 2) or to 5ul droplets for individual culture (assay 3). Drops were covered with mineral oil and

culture was performed at 38.5 °C, 5 % CO2 and maximum humidity, for up to 48 h.

2.4. Embryo culture

In assays 1 and 2, embryos were cultured in groups for 2 h or 48 h, and each embryo was imaged in separate channels (red for lipid, and green for oxidative stress). In assay 3, snapshots were taken from individual embryos at the following time points: 0 h, 2 h and 4 h; 18 h–28.5 h, every 1.5 h (8 snapshots) and at 48 h, totaling 12 snapshots of each embryo. Track of each individual embryo was kept on fixation and staining for lipids, oxidative stress, and nuclei.

2.5. Lipid and oxidative stress measurements

Oxidative stress was measured using CellRox (Thermo, C10444) assay, following manufacturer instructions. The cell-permeant dye is weakly fluorescent while in a reduced state and exhibits bright green photostable fluorescence upon oxidation by reactive oxygen species (ROS) and subsequent binding to DNA. Embryos were stained in in vitro culture droplets of SOF medium (CellRox ready-to-use solution, 1:100). After 40 min, embryos were washed and fixed in PFA 4 % overnight at 4 °C. After, Nile Red was used to quantitate neutral lipids in the embryos. Fixed embryos were stained with a Nile Red/HOECHST 33342 solution (stock 1 mg/mL in DMSO, final solution 1:100 in PBS supplemented with 1 % FCS) for 2 h. Embryos were washed 2x in PBS 1%FCS, mounted on slides and imaged in EVOS 5000 inverted microscope. For assays 1 and 2, grouped embryos were processed in 50 μl drops for staining. For assay 3, individual embryos were stained in 5 μl droplets covered with mineral oil.

2.6. Morphological parameters assessment

Snapshots were examined for reexpansion, indicated by the presence of a fluid-filled cavity, at 0, 2, and 4 h. At the 4 h mark, images were analyzed using ImageJ. The zona pellucida (ZP) was measured at its thinnest point, and the embryo area was also assessed. The presence of debris (small fragments of cytoplasm and membranes) was recorded, and any extruded cells—cells separated from the embryo into the perivitelline space—were evaluated. At the 48 h evaluation, hatching was determined by identifying any embryo projection beyond the limits of the ZP.

2.7. Statistical analysis

Linear regression was performed after residuals normality distribution and autocorrelation check. To fulfill this premise, data was transformed using log or square root, when necessary, as described below. In assay 1, data for 2 h experiment was transformed in logarithmic scale (oxidative stress and lipid levels) and linear regression was calculated initially considering oxidative stress levels as variable of interest and lipid levels and cell number as dependent variables. Since cell number was not significant, the model was adjusted for only lipid level as dependent variable. Data for 48 h experiment was transformed by square root (oxidative stress and lipid levels) and linear regression was considering oxidative stress levels as variable of interest and lipid levels as dependent variable. Graphs are presented using non-transformed data.

In assay 2, data (oxidative stress and lipid levels) was transformed by BoxCox, and multivariable linear regression was performed considering oxidative stress levels as variable of interest, lipid levels as dependent variable, and group as categorical predictor variable. The lipid fraction and nuclei fraction were analyzed by logistic regression. Lipid area, lipid levels, nuclei area, and embryo area were analyzed by T-test. To achieve normality, lipid area, nuclei area and embryo area were transformed using the square root function, while lipid levels and oxidative stress levels were transformed using the Box-Cox trasformation. Lipid intensity was analyzed using the Kruskal-Wallis test, adjusted for ties. Oxidative stress levels were analyzed using Welch corrected T-test. In assay 3, logistic regression was used to estimate hatching (the dependent variable), and linear regression was used to estimate cell number, based on predictor variables: lipid content, oxidative stress levels, zona pellucida (ZP) thickness, embryo area, presence of debris, and presence of extruded cells, for both vitrified-warmed and fresh embryos. For vitrified embryos, expansion at 0, 2 and 4 h were also included as predictor variables. All analysis were performed using R [21]. Packages used were: tidyverse [25] and lmtest [8].

3. Results

3.1. Correlation between lipid levels and oxidative stress in vitrified embryos

In assay 1, we investigated the correlation between lipid content and oxidative stress in blastocysts subjected to vitrification-warming (Fig. 1A).

Embryos stained 2 h after vitrification-warming revealed a positive correlation ($p = 0.025$) between lipid content and oxidative stress, accounting for approximately 7.8 % of the variability in oxidative stress levels (R-squared) (Fig. 1B.I). Although cell number was initially included in the model, it did not significantly affect oxidative stress levels ($p = 0.248$), leading to subsequent analyses focusing solely on lipid and oxidative stress levels. The average blastocyst cell number was 103.91 ± 30.19, lipid levels ranged from 3.80 to 71.62 (mean 25.25 ± 1.62), and oxidative stress levels ranged from 9.96 to 79.24 (mean 38.28 ± 25.25).

In a follow-up experiment, embryos stained 48 h after vitrification-warming also showed a correlation ($p < 0.001$) between lipid content and oxidative stress levels, explaining approximately 18.53 % of the variability in oxidative stress levels (R-squared) (Fig. 1B.II). A linear positive correlation was observed (Pearson coefficient = 0.42), indicating that increased lipid content is associated with higher levels of oxidative stress in vitrified bovine embryos. Lipid levels ranged from 2.84 to 35.81 (mean V = 12.95 ± 1.54, F = 15.73 ± 1.10), and oxidative stress levels ranged from 4.31 ± to 45.86 (mean V = 17.71 ± 1.38, F = 18.93 ± 1.18).

3.2. Correlation between lipid levels and oxidative stress levels and comparative analysis of lipids in fresh and vitrified embryos

In assay two, we aimed to understand the impact of vitrification on lipid content. Assessments were conducted 48 h post-warming, and factors potentially influencing lipid analysis, such as embryo size and degree of expansion, were also examined.

We observed a consistent correlation between lipid levels and oxidative stress levels (Fig. 2), similar to the previous analyses. Notably, this analysis indicated that lipid levels explained up to 51 % (multiple R-squared) of the variability in oxidative stress levels in this model.

A similar pattern was observed for both fresh ($p < 0.001$, Pearson coefficient = 0.65) and vitrified embryos ($p < 0.001$, Pearson coefficient = 0.71), with higher oxidative stress levels associated with increased lipid content. Vitrification was found to negatively affect ($p = 0.01$) lipid levels compared to the fresh control group, as shown in the subsequent group comparison analysis.

Images of blastocysts stained with Nile Red are shown in Fig. 3A. Differences in embryo size between the groups were evident: the embryo area was significantly larger ($p < 0.001$) in fresh embryos compared to vitrified embryos (Fig. 3B.I, Vitrified = 6492.50 ± 501.32, Fresh = 9921.40 ± 602.59). To account for the size difference, we analyzed lipid staining relative to embryo area. We calculated the percentage of the embryo area stained for lipids (Fig. 3B.II, Vitrified = 47.21 ± 5.53, Fresh = 57.80 ± 5.27). Given that vitrified-warmed embryos have a

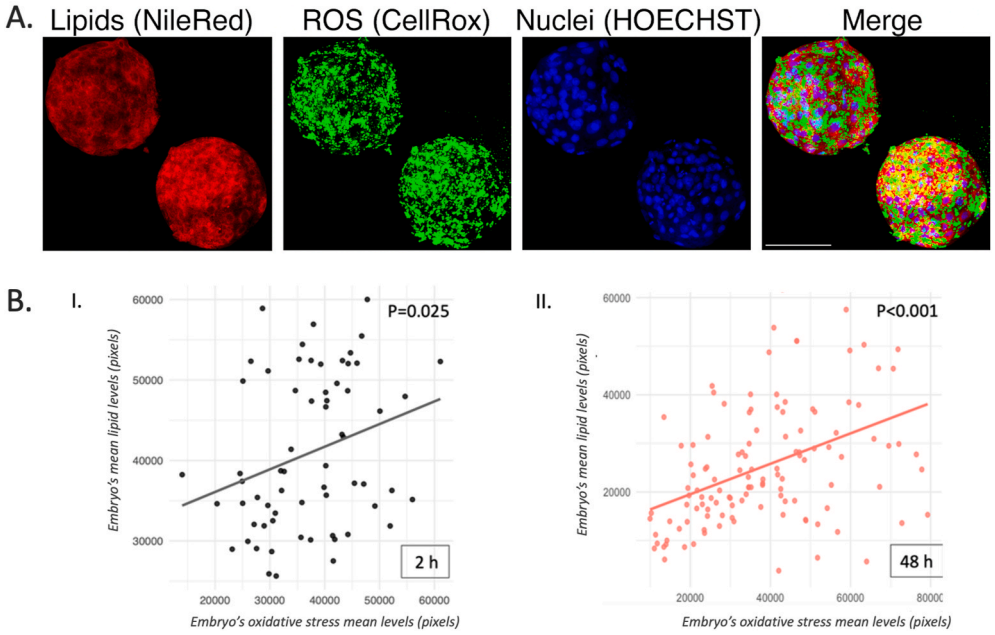


Fig. 1. Effects of lipid amount on oxidative stress levels in vitrified-warmed bovine embryos after 2 h and 48 h. A. Images of lipids (red), oxidative stress (green), and nuclei (blue) staining in vitrified embryos – note the right-side embryo displaying more lipids and more oxidative stress than left side embryo. B. Scatterplots of lipid (Nile Red staining) and oxidative stress (CellRox staining) levels are show for two separate experiments in assay 1: I. 2 h after warming (n = 64) and II. 48 h after warming (n = 122). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

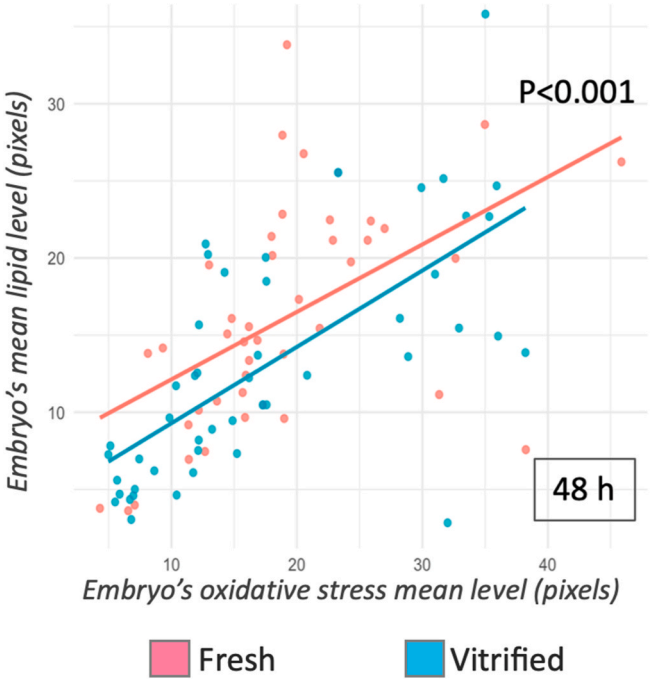


Fig. 2. Effects of vitrification-warming on lipid amount and oxidative stress on bovine embryos after 48 h. Scatterplot of lipid (Nile Red staining) and oxidative stress (CellRox staining) levels in 49 vitrified-warmed (V group) and 41 fresh (F group) grade I blastocysts. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

smaller area, this relative indicator suggests a lower quantity of lipids in vitrified-warmed blastocysts. The total lipid area (Fig. 3B.III, Vitrified = 2575.56 ± 0.81, Fresh = 5360.89 ± 0.43) was lower ($p < 0.001$) in vitrified embryos compared to fresh embryos. This absolute measure should be interpreted with caution due to the smaller embryo area in vitrified-warmed embryos, although the total lipid area was twice as high in fresh embryos, while the embryo area was only 1.5 times greater.

Additionally, staining intensity within lipid droplets was significantly higher ($p = 0.042$) in vitrified embryos (Fig. 3B.IV, Vitrified = 15.40 ± 0.25, Fresh = 13.60 ± 0.44). This measure indicates the intensity of Nile Red staining specifically for lipids, providing insights into lipid density and profile. The mean intensity of lipid staining across the entire embryo area showed a similar trend ($p = 0.058$) between the two groups (Fig. 3B.V, Vitrified = 12.95 ± 1.09, Fresh = 15.73 ± 1.11).

The percentage of embryo area stained for HOECHST, corresponding to the nuclei fraction (Vitrified = 40.08 ± 2.32, Fresh = 44.40 ± 1.49) (Fig. 3B.VI), and the total nuclei area (Vitrified = 2567.09 ± 207.08, Fresh = 4404.11 ± 196.05) (Fig. 3B.VII), were significantly larger ($p < 0.001$) in fresh embryos. The decrease in embryo area in the vitrified-warmed group may indicate either (i) reduced expansion and blastocoel filling or (ii) slower development. Given the lower nuclei area (both relative and absolute), this suggests that the reduced area might be related to a lower number of cells in the vitrified-warmed group.

Oxidative stress levels at this time point within embryos were similar ($p = 0.24$) between the control and vitrified groups (Fig. 3B.VIII, Vitrified = 17.71 ± 1.52, Fresh = 18.93 ± 1.38).

3.3. *Influence of lipid and oxidative stress levels on embryo viability following vitrification-warming*

In Assay 3, we evaluated factors affecting the developmental potential of individual embryos after vitrification and warming. Our primary objectives were to correlate lipid and oxidative stress patterns with cryosurvival and to identify predictors of viability for vitrified-warmed embryos.

Table 1 shows the variables used to predict hatching. Lipid content and oxidative stress levels had no significant impact on hatchability. Similarly, expansion at 4 h, the presence of debris, and extruded cells had non-significant coefficients and odds ratios close to 1, indicating these variables had minimal effect on the likelihood of hatching in this model.

In contrast, embryos that expanded during the warming procedure (0 h) and after 2 h had a higher chance of hatching ($p = 0.015$ and $p =$

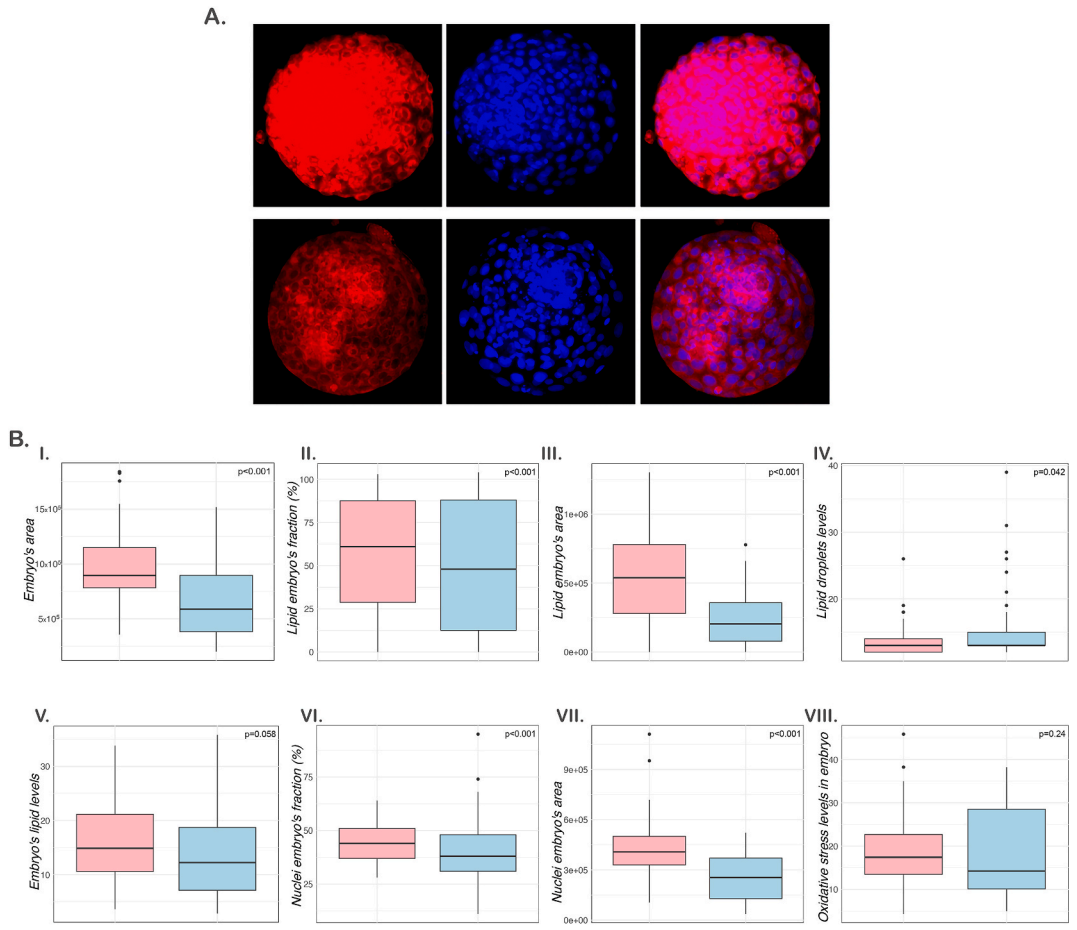


Fig. 3. Effects of vitrification-warming on lipid amount, cell number and oxidative stress of bovine embryos after 48 h. A. Images of fresh and vitrified embryos stained for Nile Red and HOECHST. B. Boxplots are shown for embryo's area (I); lipid content parameters: lipid fraction (II, the percentage of embryo stained for lipids); lipid area (III, the absolute area inside the embryo stained for lipids); lipid intensity (IV, the intensity of Nile Red staining in positive (lipid) area); and lipid level (V, the intensity of Nile Red staining in all embryo area); nuclei parameters: nuclei fraction (VI, the percentage of embryo stained for nuclei) and nuclei area (VII, the absolute are inside the embryo stained for nuclei), and oxidative stress levels (VIII, the intensity of oxidative stress staining in all embryo area) (n = 90 blastocysts, 41–49 per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.008, Odds ratio 1.30 and 1.58). This suggests that expansion at the time of warming (0 h) and after 2 h are significant predictors of hatching for vitrified embryos.

Although the coefficients for ZP thickness ($p = 0.013$) and embryo area ($p < 0.001$) are statistically significant, their practical impact is relatively small, as indicated by odds ratios close to 1. Specifically, a higher ZP thickness is associated with a lower probability of hatching, assuming all other variables are constant.

We also assessed fresh embryos for most parameters, but no significant associations were found between predictor variables and hatchability (Table 1).

Table 2 presents the results of linear regression analyses that exploring the correlation between various predictor variables, measured within the first 4 h after warming, and the subsequent cell number evaluated 48 h post-warming (equivalent to day 9 of development) for both vitrified-warmed and fresh embryos.

For vitrified-warmed embryos, lipid content and oxidative stress levels showed no significant association with embryo cell number ($p = 0.382$ and $p = 0.120$, respectively). However, there was a positive association ($p = 0.002$) between the degree of expansion at the time of warming (0 h) and the subsequent cell number. Additionally, embryo area was positively associated ($p = 0.047$) with cell number. No significant associations were found between expansion at 2 h ($p = 0.507$) or 4 h ($p = 0.618$) after warming and subsequent cell number. Likewise, ZP thickness, the presence of debris ($p = 0.121$), and the presence of extruded cells ($p = 0.092$) were not associated with cell number.

For fresh embryos, the only significant predictor was ZP thickness ($p = 0.036$), which revealed a negative association with subsequent cell number.

3.4. *Relevant predictors for the viability of vitrified-warmed embryos assessed immediately after warming*

Using the data from individual embryo assessments (Assay 3), as exemplified by the analysis of three embryos shown in Fig. 4A., this section consolidates key predictors identified through logistic regression (hatching) and linear regression (cell numbers).

Fig. 4.B.I shows a higher percentage of hatching in embryos that expanded during the warming process. A similar pattern was observed for expansion 2 h post-warming (Fig. 4.B.II). Notably, none of the embryos that failed to expand after 2 h managed to hatch. Additionally, embryos that achieved hatching later had a larger embryo area assessed 4 h after warming (Fig. 4.B.III). Conversely, ZP thickness measured at 4 h post-warming was lower in embryos that successfully hatched by the end of 48 h (Fig. 4B.IV).

Regarding cell numbers, embryos that expanded during the warming process (0 h) had higher cell counts at 48 h post-warming. Moreover, there was a positive correlation between embryo area and cell number, indicating that a larger embryo area at 4 h is associated with an increased cell number at 48 h after warming.

Table 1
Prediction of early hatching onset based on lipid content, oxidative stress, and morphological traits in bovine vitrified-warmed and fresh embryos.

Dependent variable	Predictor Variable	Coefficient	Odds Ratio	P
Hatch (V)	Lipid content	3.99e-06	1.00	0.446
	Oxidative stress levels	2.10e-06	1.00	0.583
	Expansion 0 h	**0.26**	**1.30**	**0.015**
	Expansion 2 h	**0.46**	**1.58**	**0.008**
	Expansion 4 h	0.42	1.52	0.147
	ZP thickness	**−4.24e-05**	**0.99**	**0.013**
	Embryo area	**1.90e-05**	**1.00**	**<0.001**
	Debris	0.08	1.09	0.577
	Extruded cells	−0.05	0.94	0.620
Hatch (F)	Lipid content	9.53e-06	1.00	0.099
	Oxidative stress levels	7.70e-06	1.00	0.141
	ZP thickness	−2.57e-05	0.99	0.228
	Embryo area	7.12e-06	1.00	0.053
	Debris	−0.24	0.78	0.183
	Extruded cells	−0.15	0.85	0.344

The table presents the results of a logistic regression analysis assessing potential predictors of the outcome variable "hatch" for vitrified-warmed (V) and fresh control (F) embryos. Hatching, was assessed 48 h after warming (day 9). Predictor variables that are statistically significant are highlighted in bold and underlined. Embryo expansion was measured at 0, 2, and 4 h post-warming. Zona pellucida (ZP) thickness, embryo area, debris, and extruded cells were evaluated 4 h after warming. Lipid content and oxidative stress levels were quantified 48 h after warming.

Table 2
Linear regression analysis among potential predictors and outcome variable "cell number in 48 h" in vitrified-warmed embryos.

Dependent variable	Predictor Variable	Coefficient	*Adjusted* R^2	P
Cell number (48 h, V)	Lipid content	2.17e-05	−0.003	0.382
	Oxidative stress levels	2.99e-05	0.022	0.120
	Expansion 0 h	**1.65**	**0.119**	**0.002**
	Expansion 2 h	0.60	−0.008	0.507
	Expansion 4 h	−0.81	−0.011	0.618
	ZP thickness (4 h)	−7.59e-05	−0.004	0.382
	Embryo area (4 h)	**4.71e-05**	**0.045**	**0.047**
	Debris	−1.26	0.022	0.121
	Extruded cells	−0.94	0.028	0.092
Cell number (48 h, F)	Lipid content	2.44e-05	−0.016	0.429
	Oxidative stress levels	6.19e-05	4.03e-05	0.329
	ZP thickness (4 h)	**−0.0003**	**0.154**	**0.036**
	Embryo area (4 h)	0.38	0.060	0.135
	Debris	−0.84	−0.020	0.480
	Extruded cells	−0.22	−0.040	0.841

4. Discussion and conclusion

In this study, we examined the impact of lipid quantity on oxidative stress and the viability of vitrified-warmed blastocysts using an individual embryo analysis approach. Our findings revealed that vitrified-warmed embryos (i) had lower lipid content than fresh counterparts following 48 h of culture, and that (ii) neutral lipid levels in these embryos were correlated with oxidative stress. However, (iii) neither lipid levels nor oxidative stress levels were reliable predictors of cryosurvival. We also found that (iv) expansion at the time of warming and embryo area are good indicators of increased cryosurvival potential for vitrified-warmed embryos.

Our first key observation was the reduction in lipid levels found in vitrified embryos compared to their fresh counterparts. This finding suggests increased lipid metabolism aimed at restoring the embryo's lifecycle post-vitrification-warming, as no specific treatment was performed to reduce lipid content and embryos were randomly assigned to either the fresh or vitrified groups. Indeed, profiles of phosphatidylcholines, sphingomyelins and triacylglycerols are altered following cryopreservation in bovine blastocysts, reflecting a metabolic response [12]. Albeit not the primary focus of the present study, Romek et al. [22] have shown that Nile Red emission spectra differ among triglycerides, phospholipids, and cholesterol, which could relate to changes in lipid droplet levels observed in our study. Thus, our data suggest that lipid metabolism is affected by vitrification-warming, by reducing the quantity of lipid droplets (indicated by a smaller stained area and total lipid area) and potentially altering the lipid profile (reflected by higher staining intensity within droplets). While evidence suggests lipid profile may impact cryosurvival [11,12], this aspect was not examined in our study.

Several studies suggest that the accumulation of lipid droplets may exert a detrimental effect on cryosurvival outcomes. However, attempts to reduce lipid content in embryos do not consistently improve cryosurvival rates [15]. The variability in findings, where lipid reduction is associated with increased cryosurvival in some studies [2,24] but not others [7], may be related to the role of lipid droplets. It is important to note that our conditions, which align with common commercial practices in Brazil — such as high serum levels during the IVM step and high oxygen levels — are associated with increased oxidative stress.

Although high lipid content is correlated with apoptosis in vitrified blastocysts [19,24], it is important to note that neutral lipids serve as major intracellular energy storage molecules [3], which might influence embryo recovery post-vitrification. There is no standard lipid droplet amount in bovine embryos due to a high physiological variance, which makes it difficult to determine what amount is normal or aberrant. Nevertheless, lipid accumulation may indicate environmental stress during embryo culture [7]. Increased lipid content has been correlated with apoptosis in vitrified blastocysts [19,24]. Excessive glycolysis stimulation may contribute to lipid accumulation and changes in lipid metabolism, along with oxidative stress and mitochondrial dysfunction [5]. Additionally, lipid droplets, particularly large ones, are associated with higher levels of immature mitochondria [1]. Thus, higher lipid levels may reflect underlying issues leading to cell death and decreased embryo viability, such as enhancement of reactive oxygen species (ROS) and reduced mitochondrial activity in bovine embryos [20].

Indeed, our findings reveal a correlation between lipid levels and oxidative stress levels in vitrified embryos, both immediately after warming and 48 h later. This association mirrors that observed in fresh embryos, albeit with increased magnitude following vitrification. This suggests that in vitrified-warmed embryos, lipid reserves explain oxidative stress more than in fresh embryos. This could be due to increased lipid utilization, as evidenced by the reduced lipid content. Our data suggests that vitrification and warming lead to increased lipid utilization, which is reflected in the lower lipid content. Reactive oxygen species (ROS) are molecules with one or more unpaired electrons, rendering them highly reactive as they seek electron stability by oxidizing other molecules, and lipids are among the most oxidized macromolecules. Lipid peroxidation induces cellular damage, as peroxyl radicals react with polyunsaturated fatty acids to form hydroperoxides and alkyl radicals, initiating a chain reaction that compromises membrane fluidity and function [10]. Previous experiments in our model have shown increased oxidative stress in embryos immediately post-vitrification compared to fresh counterparts (data not shown). This outcome is expected, as embryos must be metabolically active to reestablish cell cycles after cryopreservation. This is reflected by decreased lipid levels and increased ROS production, since metabolically active embryos generate more ROS than quiescent counterparts [16].

Interestingly, we found similar levels of oxidative stress in both fresh and vitrified embryos 48 h post-vitrification, suggesting that vitrified

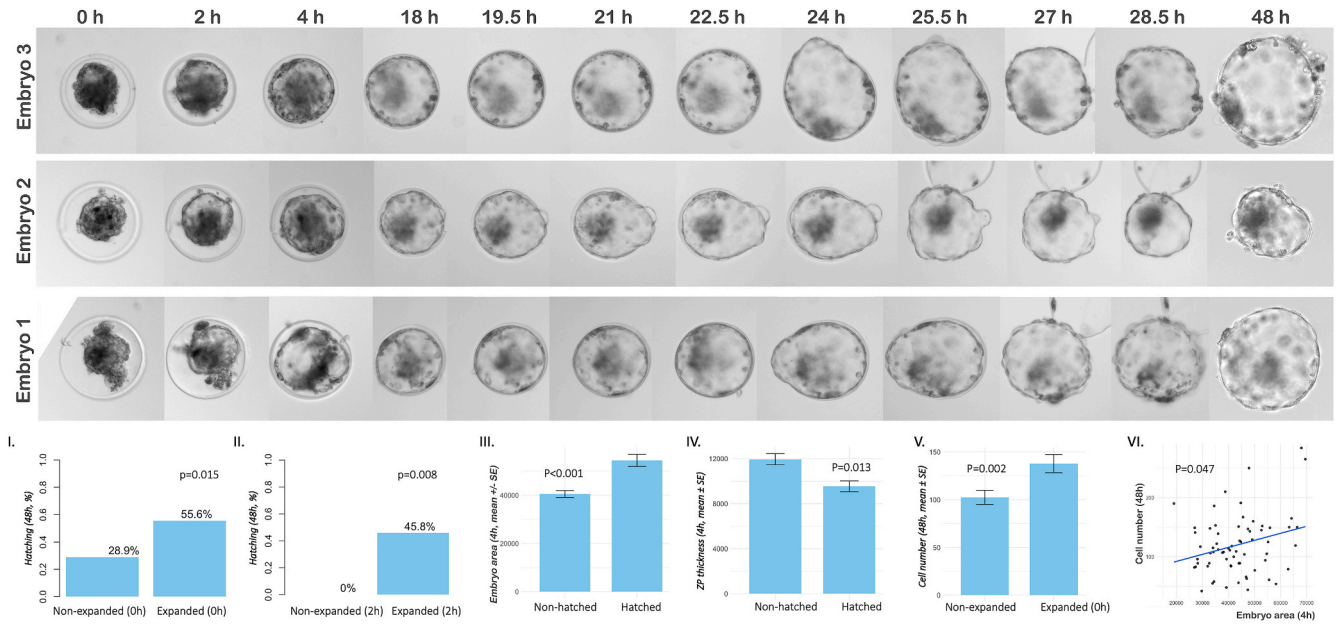


Fig. 4. Early prediction of vitrified-warmed blastocysts performance. A. Snapshots of 0 h, 2 h and 4 h (early evaluation); following hatching timelapse from 18 to 29.5 h (1.5 h intervals); and 48 h (final evaluation) after warming, showing hatching of a random sample of three vitrified-warmed embryos. B. Statistically relevant predictor variables for hatching and cell number increase in vitrified-warmed bovine embryos (I,II). Percentage of hatching regarding expansion at 0 h (I) and 2 h (II) after warming. Embryo area (III) and ZP thickness (IV) in non-hatched and hatched embryos. (V, VI) Cell number in non-expanded and expanded embryos at 0 h (V) and relationship between embryo area and cell number (VI) (n = 99 blastocysts, 76 vitrified-warmed and 23 fresh) (n = 99 blastocysts, 76 vitrified-warmed and 23 fresh).

embryos can effectively regulate oxidative stress within this timeframe. Despite morphological differences, vitrified embryos manage to achieve oxidative stress levels comparable to fresh counterparts. However, the increase in metabolic activity during warming phase suggests a complex relationship between oxidative stress and lipid accumulation. Whether this indicates balanced production of oxidized compounds or fewer radicals due to lower lipid amount in vitrified embryos is unclear. Thus, perhaps at other developmental stages, this relationship may impact cryosurvival.

No morphological parameter related to cryosurvival, including hatchability and cell number, was associated with lipid levels or oxidative stress in individual embryos in our study. Coefficients and odds ratios for these traits were generally low and statistically insignificant, suggesting that other factors play a more substantial role. Significant predictors of hatchability in vitrified embryos include expansion at 0 h and 2 h, zona pellucida thickness, and embryo area, with early expansion linked to increased hatchability (in terms of odds ratio). Increased cell numbers at 48 h could be predicted based on early expansion (0 h) and embryo area. Additionally, zona pellucida thickness at 4 h was negatively associated with cell number in fresh embryos. As expected, fresh embryos had more cells compared to vitrified embryos. However, the degree of expansion, assessed by the percentage of the embryo covered with nuclei staining, did not decrease in the vitrified group. Furthermore, consistent with the damage caused by vitrification, embryo size was larger in fresh embryos.

In summary, our results suggest a decrease in lipid levels following the vitrification-warming procedure and demonstrate an individual association between lipid content and oxidative stress in vitrified embryos. However, no direct effect of lipid quantity or oxidative stress on the survivability of vitrified embryos was observed, at least during the initial 48 h post-warming. Expansion at the time of warming and embryo area can be considered good indicators of increased cryosurvival chances for vitrified-warmed embryos, while stress parameters such as cellular debris and extruded cells had no effect on hatchability or cell numbers.

CRediT authorship contribution statement

Clara Slade Oliveira: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Viviane Luzia da Silva Feuchard:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carolina Romano Capobiango Quintão:** Writing – review & editing, Formal analysis, Data curation. **Leticia Zoccolaro Oliveira:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Naiara Zoccal Saraiva:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve scientific language. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

The authors declare no competing financial or personal interests that could have influenced in this study.

Acknowledgements

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) as Master's Degree grant (Finance Code 001); Brazilian Agricultural Research Corporation (Embrapa; Grant 12.13.06.020.00.00) and FAPEMIG (APQ 02041–22).

References

[1] H. Abe, S. Yamashita, T. Satoh, H. Hoshi, Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media, Mol. Reprod. Dev. 61 (1) (2002) 57–66.

[2] M.F. Accorsi, B.C.D.S. Leão, N.A.D.S. Rocha-Frigoni, S.H.V. Perri, G.Z. Mingoti, Reduction in cytoplasmic lipid content in bovine embryos cultured in vitro with linoleic acid in semi-defined medium is correlated with increases in cryotolerance, Zygote 24 (2016).

[3] P.S. Bertevello, A.P. Teixeira-Gomes, A. Seyer, A.V. Carvalho, V. Labas, M. C. Blache, C. Banliat, L.A.V. Cordeiro, V. Duranthon, P. Papillier, V. Maillard, S. Elis, S. Uzbekova, Lipid identification and transcriptional analysis of controlling enzymes in bovine ovarian follicle, Int. J. Mol. Sci. 19 (10) (2018) 3261.

[4] C.M.H. Combelles, M.L. Hennet, Oxidative stress in assisted reproductive technologies, in: Studies on Women's Health, 2013.

[5] L.R.O. Dias, I. Pivato, M.A.N. Dode, Change in energy metabolism of in vitro produced embryos: an alternative to make them more cryoresistant? Semina Ciências Agrárias 38 (2017).

[6] C. Diez, Y. Heyman, D. Le Bourhis, C. Guyader-Joly, J. Degrouard, J.P. Renard, Delipidating in vitro-produced bovine zygotes: effect on further development and consequences for freezability, Theriogenology 55 (2001).

[7] E. Held-Hoelker, S.L. Klein, F. Rings, D. Salilew-Wondim, M. Zidane, C. Neuhoff, D. Tesfaye, K. Schellander, M. Hoelker, Cryosurvival of in vitro produced bovine embryos supplemented with L-Carnitine and concurrent reduction of fatty acids, Theriogenology 96 (2017).

[8] T. Hothorn, A. Zeileis, Diagnostic checking in regression relationships, R. News 2 (2011).

[9] B. Kadenbach, S. Arnold, I. Lee, M. Hüttemann, The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases, Biochim. Biophys. Acta Bioenerg. 1655 (2004).

[10] J.A. Keane, A.D. Ealy, An overview of reactive oxygen species damage occurring during in vitro bovine oocyte and embryo development and the efficacy of antioxidant use to limit these adverse effects, Animals 14 (2024).

[11] B.C.S. Leão, N.A.S. Rocha-Frigoni, E.C. Cabral, M.B. Coelho, C.R. Ferreira, M. N. Eberlin, M.F. Accorsi, T. Nogueira, G.Z. Mingoti, Improved embryonic cryosurvival observed after invitro supplementation with conjugated linoleic acid is related to changes in the membrane lipid profile, Theriogenology 84 (2015).

[12] B.C.S. Leão, N.A.S. Rocha-Frigoni, É. Nogueira, E.C. Cabral, C.R. Ferreira, M. N. Eberlin, M.F. Accorsi, T.V. Neves, G.Z. Mingoti, Membrane lipid profile of in vitro-produced embryos is affected by vitrification but not by long-term dietary supplementation of polyunsaturated fatty acids for oocyte donor beef heifers, Reprod. Fertil. Dev. 29 (2017).

[13] R.F. Leite, K. Annes, J. Ispada, C.B. De Lima, É.C. Dos Santos, P.K. Fontes, M.F. G. Nogueira, M.P. Milazzotto, Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos, Oxid. Med. Cell. Longev. 2017 (2017).

[14] S. Madrid Gaviria, S.A. Morado, A.L. Herrera, G.R. Betancur, R.A.U. Álvarez, J. E. Zuluaga, P.D. Cética, Resveratrol supplementation promotes recovery of lower oxidative metabolism after vitrification and warming of in vitro -produced bovine embryos, Reprod. Fertil. Dev. 31 (2019).

[15] F. de A. Melo-Sterza, R. Poehland, Lipid metabolism in bovine oocytes and early embryos under in vivo, in vitro, and stress conditions, Int. J. Mol. Sci. 22 (2021).

[16] S.G. Moore, A. O'Gorman, L. Brennan, T. Fair, S.T. Butler, Follicular fluid and serum metabolites in Holstein cows are predictive of genetic merit for fertility, Reprod. Fertil. Dev. 29 (2017).

[17] C.S. Oliveira, V.L.S. Feuchard, C. Freitas, P.M.S. Rosa, A.J.R. Camargo, N. Z. Saraiva, In-straw warming protocol improves survival of vitrified embryos and allows direct transfer in cattle, Cryobiology 97 (2020).

[18] C.S. Oliveira, N.Z. Saraiva, M.R. de Lima, L.Z. Oliveira, R.V. Serapião, J.M. Garcia, C.A.V. Borges, L.S.A. Camargo, Cell death is involved in sexual dimorphism during preimplantation development, Mech. Dev. 139 (2016) 42–50.

[19] C.S. Oliveira, V.L. Da Silva Feuchard, S.C. De Souza Marques, N.Z. Saraiva, Modulation of lipid metabolism through multiple pathways during oocyte maturation and embryo culture in bovine, Zygote 30 (2022).

[20] S. Prastowo, A. Amin, F. Rings, E. Held, D.S. Wondim, A. Gad, C. Neuhoff, E. Tholen, C. Looft, K. Schellander, D. Tesfaye, M. Hoelker, Fateful triad of reactive oxygen species, mitochondrial dysfunction and lipid accumulation is associated with expression outline of the AMP-activated protein kinase pathway in bovine blastocysts, Reprod. Fertil. Dev. 29 (2017).

[21] R Core Team., R: A Language and Environment for Statistical Computing, vol. 13, R Foundation for Statistical Computing, Vienna, Austria, 2023. Open J Stat.

[22] M. Romek, B. Gajda, E. Krzysztofowicz, M. Kepczynski, Z. Smorag, New technique to quantify the lipid composition of lipid droplets in porcine oocytes and pre-implantation embryos using Nile Red fluorescent probe, Theriogenology 75 (2011).

[23] M.J. Sudano, D.M. Paschoal, T. Da Silva Rascado, L.F. Crocomo, L.C.O. Magalhães, A.M. Junior, R. Machado, F. Da Cruz Landim-Alvarenga, Crucial surviving aspects for vitrified in vitro-produced bovine embryos, Zygote 22 (2014).

[24] M.J. Sudano, D.M. Paschoal, T. da Silva Rascado, L.C.O. Magalhães, L.F. Crocomo, J.F. de Lima-Neto, F. da Cruz Landim-Alvarenga, Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification, Theriogenology 75 (7) (2011) 1211–1220.

[25] H. Wickham, M. Averick, J. Bryan, W. Chang, L. McGowan, R. François, G. Grolemund, A. Hayes, L. Henry, J. Hester, M. Kuhn, T. Pedersen, E. Miller, S. Bache, K. Müller, J. Ooms, D. Robinson, D. Seidel, V. Spinu, K. Takahashi, D. Vaughan, C. Wilke, K. Woo, H. Yutani, Welcome to the tidyverse, J. Open Source Softw. 4 (2019).

[26] A.M. Zolini, E. Carrascal-Triana, A. Ruiz de King, P.J. Hansen, C.A. Alves Torres, J. Block, Effect of addition of L-carnitine to media for oocyte maturation and embryo culture on development and cryotolerance of bovine embryos produced in vitro, Theriogenology 133 (2019) 135–143.