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Effect of delipidant agents during in vitro culture on the development, lipid content, gene expression and cryotolerance of bovine embryos

Luzia Renata Oliveira Dias[1] | Ligiane Oliveira Leme[2] | José Felipe Warmling Sprícigo[3] | Ivo Pivato[1] | Margot Alves Nunes Dode[1,4]

[1]University of Brasília, Brasília, DF, Brazil

[2]Federal University of Espírito Santo, Alegre, ES, Brazil

[3]Department of Animal Bioscience, University of Guelph, Guelph, ON, Canada

[4]EMBRAPA Genetic Resources and Biotechnology, Brasília, DF, Brazil

Correspondence
Margot Alves Nunes Dode, University of Brasília, Brasília, DF, Brazil.
Email: margot.dode@embrapa.br

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Abstract

In vitro produced embryos are still sensitive to the freezing process which can be explained, in part, by the high-lipid accumulation that characterizes these embryos. Therefore, we aimed to evaluate the effect of delipidating agents, L-carnitine and the trans-10 cis-12 conjugated linoleic acid (CLA) isomer, on blastocyst development, lipid content, gene expression and cryotolerance when added to embryo culture media. Embryos were cultured in four different media: T1: control (n = 616), synthetic oviduct fluid (SOF) media with 5% foetal bovine serum (FBS); T2: L-carnitine (n = 648), SOF medium with 5% FBS and 0.6 mg/ml of L-carnitine; T3: CLA (n = 627), SOF medium with 5% FBS and 100 μM trans-10 cis-12 CLA; and T4: L-carnitine + CLA: (n = 597), SOF medium with 5% FBS plus 0.6 mg/ml L-carnitine and 100 μM trans-10 cis-12 CLA. Supplementation of culture medium with either or both delipidating agents reduced (p < .05) blastocyst rate on D7 (T1 = 49 ± 3.5; T2 = 39 ± 3.0; T3 = 42 ± 3.9 and T4 = 39 ± 3.9), but did not affected gene expression (p > .05). Although embryos cultured in the presence of L-carnitine contained fewer (p < .05) lipid droplets than the control embryos, they showed a lower re-expansion rate 24 hr post-thaw than those (p < .05). In conclusion, although L-carnitine reduced the amount of lipids in cultured embryos, the use of L-carnitine and CLA during in vitro culture was not able to improve the embryo production and the response to cryopreservation.

KEYWORDS

cattle, CLA, cryopreservation, L-carnitine

1 | INTRODUCTION

Cryopreservation is crucial to overcome the logistic problems of commercial in vitro embryo production (IVP), especially due to the large number of embryos resulting from an ovum pick-up session and the limitation of recipient availability. In addition, this technique is essential for the storage of gametes and formation of germplasm banks, for conservation of breeds and endangered species (Mariante, Albuquerque, & Ramos, 2011), as well as for commercial purposes.

However, IVP embryos are still sensitive to the freezing process; reduced cryotolerance can be explained in part, by the high-lipid accumulation that characterizes these embryos. There is evidence that at least four classes of lipids affect post-cryopreservation embryo survival: triglycerides (TAG), which represent most of the lipids present in the cytoplasm, free fatty acids (FA), cholesterol and phospholipids (Sudano et al., 2013). Thus, the correlation between the embryo lipid content and its cryotolerance has been widely demonstrated (Absalón Medina et al., 2014; Batista et al., 2014; Leão et al.,

2015, 2017; Paschoal et al., 2017; Pereira et al., 2007; Sudano et al., 2011, 2012; Takahashi et al., 2013).

A variety of studies has been focused on reducing lipid content, aiming to increase embryo cryotolerance (Absalón Medina et al., 2014; Batista et al., 2014; Leão et al., 2015, 2017; Paschoal et al., 2017; Pereira et al., 2007; Sudano et al., 2011, 2012; Takahashi et al., 2013). Thus, various substances have been used to regulate lipid metabolism during in vitro culture, such as forskolin (Men, Agca, Riley, & Critser, 2006; Paschoal et al., 2014, 2017; Sanches et al., 2013), phenazine ethosulfate (Barcelo-Fimbres & Seidel, 2007; Sudano et al., 2011), L-carnitine and conjugated linoleic acid isomers (CLA) (Absalón Medina et al., 2014; Batista et al., 2014; Takahashi et al., 2013). Among those, L-carnitine and trans-10, cis-12-CLA are potential candidates for use in embryo culture, since they have demonstrated lipid-modulating activity without affecting embryo development and subsequently improving the cryotolerance of IVP embryos (Chankitisakul, Somfai, Inaba, Techakumphu, & Nagai, 2013; Takahashi et al., 2013).

L-Carnitine is a soluble molecule that acts as a β-oxidation cofactor in lipid metabolism and is responsible for the transportation of FA into the mitochondria to generate adenosine triphosphate (ATP); in addition, it has demonstrated antioxidant activity (Abdelrazik, Sharma, Mahfou, & Agarwal, 2009; Mingorance, Rodriguez-Rodriguez, Justo, Herrera, & Sotomayor, 2011; Sutton-Mcdowall, Feil, Robker, Thompson, & Dunning, 2012). Several studies have demonstrated that its presence in maturation medium for porcine and bovine oocytes or in bovine embryo culture medium reduced lipid accumulation and altered lipid droplet distribution (Chankitisakul et al., 2013; Somfai et al., 2011; Sutton-Mcdowall et al., 2012) increased the survival rate after cryopreservation (Takahashi et al., 2013).

Conversely, CLA has been reported to reduce expression of the gene encoding the protein lipase (LDL), which is responsible for the hydrolysis extracellular TAGs and releasing FA (Baumgard, Corl, Dwyer, Saebø, & Bauman, 2000). Thus, studies have demonstrated that the addition of the trans-10, cis-12 CLA isomer decreased the lipid content and increased the cryotolerance of IVP embryos (Batista et al., 2014; Matos et al., 2015).

Based on these data, we hypothesized that the association of both molecules during in vitro culture would decrease the lipid content in blastocysts at day 7, increasing embryo cryotolerance without affecting embryo development. Therefore, our first goal was to evaluate the effect of L-carnitine and/or CLA on embryo development, lipid content and quality in in vitro culture. We investigated triglyceride storage as lipid droplets and the expression of genes related to embryo metabolism. Finally, we studied the resistance of blastocysts to cryopreservation by the slow-freezing method.

2 | MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich, unless otherwise stated.

This article does not require CEUA approval to be performed, and treating ovaries from a slaughterhouse, in accordance with Arouca Law no. 11,794 of 8 October 2008, establishes that experiments are procedures performed on live animals, and according to art 3°, sole paragraph, it is not considered experimental non-experimental interventions related to farming practices.

2.1 | Experimental design

Four experiments were performed to evaluate the effects of delipidating substances, L-carnitine and CLA, on the quantity, quality, lipid content and gene expression of IVP bovine embryos. The concentrations of delipidating substances used for IVC in all experiments were 3.03 mM (0.6 mg/ml) L-carnitine (Takahashi et al., 2013) and 100 μM CLA (Batista et al., 2014).

In the first experiment, we evaluated the effect of the two delipidating agents on embryo production and development kinetics. In total, 13 replicates were performed with 2.448 grades 1 and 2 COCs. After 18 hr of fertilization, the presumptive zygotes were removed from fertilization drops and distributed across four treatments: treatment 1 (T1—control): embryos were cultured in synthetic oviduct fluid (SOF) medium supplemented with 5% foetal bovine serum (FBS); treatment 2 (T2—L-carnitine): embryos were cultured in SOF medium supplemented with 5% FBS and L-carnitine; treatment 3 (T3—CLA): embryos were cultured in SOF medium supplemented with 5% FBS and CLA; and treatment 4 (T4—L-carnitine + CLA): SOF medium supplemented with 5% FBS, L-carnitine and CLA. Embryos were evaluated on D2 for cleavage and on D6 and D7 for Bx rate.

In the second experiment, embryos were cultured under the same conditions described above and evaluated for lipid content. Four replicates were carried out. On D7, expanded Bx was fixed and stored for further evaluation with Sudan Black B, a lipophilic dye. The number of embryos evaluated in the control, L-carnitine, CLA and L-carnitine + CLA groups was 46, 36, 37 and 35, respectively.

For the third experiment, the expression of genes related to lipid metabolism (CPT2; PLIN2), apoptosis (CASP3; CASP8, BCL2L1), cell proliferation and differentiation (FOSL1), stress (HPSA5; HPSA1A, SOD2) and cell damage repair (MSH6) was quantified on D7, in expanded Bx. Four pools of 18–20 expanded Bx (D7) from each group were used.

Finally, in the fourth experiment, embryos produced in the presence of delipidating agents were evaluated for their response to cryopreservation. On D7 of culture, embryos from each treatment group at the Bx stage were frozen by the slow-freezing method. In the fresh control group, the embryos were maintained in four-well plates containing holding medium under a heating plate at 37°C until thawed and then transferred to culture dishes. All groups were placed back into SOF medium supplemented with 5% FBS and cultured for an additional 48 hr. Re-expansion and hatch rates were evaluated 24 and 48 hr after thawing.

2.2 | In vitro embryo production

Ovaries from crossbred cows were collected at the local slaughterhouse and transported to the laboratory in saline solution (0.9% NaCl), supplemented with penicillin (100 μg/ml) and streptomycin (50 μg/ml) at 36°C. Cumulus oocyte complexes (COCs) were aspirated from ovarian follicles with a diameter of 3.0–8.0 mm, and COCs presenting three or more layers of cells and homogeneous cytoplasm were selected. Groups of up to 30 COCs were transferred to 150 μl droplets of maturation medium, covered with silicone oil and cultured for 22–24 hr at 38.5°C and 5% CO_2. The maturation medium was composed of TCM 199 Earl's salts (Gibco) supplemented with 10% FBS (Gibco), 12 IU/ml luteinizing hormone, 0.01 IU/ml follicle-stimulating hormone, 0.1 mg/ml L-glutamine, 1 μM pyruvate, 1 μM cysteamine and 0.075 mg/ml amikacin sulphate. To fertilization, frozen semen from a Nelore bull, with known fertility, was thawed at 37°C for 30 s in a water bath and sperm cells were selected via a Percoll (GE Healthcare) gradient (Machado et al., 2009). After centrifugation, the pellet was removed and centrifuged again for 5 min in TALP medium (Parrish, Krogenaes, & Susko-Parrish, 1995) supplemented with 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine and 10 μg/ml heparin, 700G. The resulting pellet was re-suspended in fertilization medium (FEC) and added to the fertilization drop at a final concentration of 1×10^6 spermatozoa/ml. Spermatozoa were co-incubated with matured oocytes for 18 hr, and then, presumptive zygotes were washed and transferred to 150 μl drops of SOF medium (Holm, Booth, Schmidt, Greve, & Callesen, 1999), supplemented with essential and non-essential amino acids, 0.34 mM sodium tri-citrate, 2.77 mM myo-inositol and 5% SFB (Invitrogen) at 38.5°C, 5% CO_2. Embryos were evaluated on Day (D) 2 for cleavage and on D6 and D7 for blastocyst rate. Embryos were classified by stage of development, and the expanded Bx on D7 was washed three times in phosphate-buffered saline (PBS) free of calcium and magnesium and stored in RNAlater Stabilization Solution (Ambion, Life Technologies) at −20°C until RNA extraction.

2.3 | Evaluation of lipid content in embryos

Lipids were quantified using Sudan Black B stain, as described by Sudano et al. (2012), with minor modifications. First, embryos at the Bx stage were washed in PBS solution, transferred to microtubes containing 100 μl of fixation solution (10% formaldehyde in PBS, pH 7.4) and stored at 7°C until the day of evaluation. For staining, embryos were washed twice in distilled water with 0.05% polyvinyl alcohol (PVA) and then transferred to a drop of 50% ethanol in distilled water for 2 min. Then, embryos were transferred to 20 μl drops of Sudan Black (1% v/v) in 70% ethanol, where they remained for 1–2 min. To remove excess dye, the embryos were washed three times (5 min each) in 50% ethanol, followed by a 5-min wash in distilled water with 0.05% PVA. Then, embryos were mounted on a slide in 10 μl drops of distilled water with 0.05% PVA, covered with a cover slip and evaluated under an optical microscope at 20× magnification. Images of each embryo were stored and evaluated using Image J 1.41 software (Wayne Rasband, National Institutes of Health). For the analysis, coloured images were converted to a grey scale. Embryos were delimited to determine the area (μm^2) and mean grey intensity (arbitrary unit), and the grey intensity per area was calculated (arbitrary units/μm^2).

2.4 | Gene expression analysis

Four pools of 18–20 Bx (D7) from each group were used. Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen®) according to the manufacturer's instructions (with minor modifications). The total volume of isolated RNA was used for cDNA synthesis using First-Strand cDNA Synthesis (Invitrogen)—SuperScript® III (200 U/μl) and primer Oligo-dT (0.5 μg/μl) in a final volume of 40 μl. Reactions were performed at 65°C for 5 min and at 50°C for 50 min, followed by inactivation of the enzyme at 85°C for 5 min. qPCR analysis was performed using Fast Sybr Green Master Mix (Applied Biosystems). Reactions were optimized to promote maximal amplification efficiency for each gene (90%–110%), using calculations obtained with relative standard curves in the 7500 2.0.3 program (Applied Biosystems). Each sample was analysed in triplicate, and the specificity of each PCR product was determined by melting curve analysis and the size of agarose gel amplicons. Reactions were performed in a final volume of 25 μl using cDNA corresponding to 0.5 of an embryo per reaction. PCR cycles were performed as follows: 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 10 s and then annealing at 60°C for 30 s. Primer details and the amplicon size for each gene are listed in Table 1.

Expression of the following genes was quantified by qPCR: perilipin 2 (PLIN2) and carnitine palmitoyltransferase 2 (CPT2), related to lipid storage; BCL2-like 1 (BCL2L1), apoptosis cysteine peptidase 3 (CASP3) and apoptosis cysteine peptidase 8 (CASP8), related to apoptosis; FOS-like 1 transcription factor subunit (FOSL1), related to the proliferation, differentiation and transformation of cells; heat shock protein family A (Hsp70) member 5 (HSPA5), heat shock protein family A member 1A (Hsp70) (HSPA1A) and superoxide dismutase 2 (SOD2), related to thermal stress; and the DNA mismatch repair protein MSH (MSH6) gene, related to cell damage repair.

The expression of three constitutively expressed genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and peptidylprolyl isomerase A, was analysed by the GeNorm program, which indicated GAPDH to be the most stable gene. This gene was therefore used as a reference for data normalization. The relative expression of each gene was calculated using the ΔΔCt method with efficiency correction by the Pfaffl method (Pfaffl, 2001).

2.5 | Slow freezing and survival of embryos after thawing

On D7, embryos were morphologically evaluated to determine development stage according to the International Embryo Technology

TABLE 1 Specific primers used to amplify gene fragments for real-time qPCR analysis

Genes	Primer sequences	Amplicon size (bp)	Primer concentration (nM)	GenBank access number
GAPDH	F: GGC GTG AAC CAC GAG AAG TAT AA R: CCC TCC ACG ATG CCA AAG T	118	300	NM_001034034.2
CASP3	F: GCC CAG GAC TTT AGC AGT CA R: AAA TGT GAG CGC CTT TGT T	185	250	NM_001077840.1
CASP8	F: CAG AAC AGA TGG AAG CCT AT R: GGT TAG GAT GGT CAG AAT GT	209	250	NM_001045970.2
FOSL1	F: GCT TCC TAG TAG AGC CAA AG R: GAA GAG GTG ATG AAG ACC ATA G	200	300	NM_001205985.1
HSPA5	F: CGT GGC CAC TAA TGG AGA TAC R: CTC TGT TGT CCT TCC GAA CAT	119	300	NM_001075148.1
HSPA1A	F: CAA GAT CAC CAT CAC CAA CG R: AAA TCA CCT CCT GGC ACT TG	219	300	NM_174550.1
SOD2	F: TTG CTG GAA GCC ATC AAA CGT GAC R: AAT CTG TAA GCG TCC CTG CTC CTT	135	300	NM_201527
CPT2	F: TCCTGGATCAAGATGGGAAC R: GTGGGACAGGTGGACAAAGT	254	200	NM_001045889.2
PLIN2	F: CGG CTA CGA TGA TAC AGA TG R: TGC GAA ACA CAG AGT AGA TG	200	300	NM_173980.2
MSH6	F: CCC AGG TGC TTA AAG GTA TG R: GGA CCA TGT CAG AAT CCA AG	186	300	NM_001192737.1
BCL2-L1	F: GAGATGCAGGTATTGGTGAG R: GGTCAGTGTCTGGTCATTTC	244	250	NM_001077486.2

Abbreviations: F, primer forward; R, primer reverse.

Society (IETS) manual (Robertson & Nelson, 1998). For freezing, embryos in the Bx stage were washed in PBS and dehydrated in a solution to 1.5 M ethylene glycol drops, loaded into 0.25 ml straws and frozen in an automatic freezing device (FREEZE CONTROL®, Model CL-863 System, Cryologic). The freezing device was set to a freezing rate of 0.5°C per minute, from −6 to −35°C. After reaching −35°C, the straws were immersed in liquid nitrogen. To thaw, each straw was kept in air for 6 s and for 20 s in a water bath at 30°C. Then, the embryos were washed in PBS and transferred back to the culture drop corresponding to the experimental group. The re-expansion and hatching of embryos were assessed 24 and 48 hr post-thawing. The control groups for both treatments were maintained on the bench in holding medium during the freeze–thaw process, transferred back to the culture drop and subsequently cultured for an additional 24 and 48 hr.

2.6 | Statistical analysis

Statistical analyses were conducted using Prophet version 5.0 (BBN Systems and Technologies, 1997). Data for lipid quantification were compared using one-way analysis of variance (ANOVA), and groups were compared using the Newman–Keuls test. The chi-square test was used to analyse cleavage, blastocyst, re-expansion and hatching rates. The relative expression of each gene was calculated using one-way ANOVA. For all analyses, a significance level of $p < .05$ was considered.

3 | RESULTS

3.1 | Effect of delipidating substances, L-carnitine and trans-10 cis-12 CLA, during in vitro culture on embryo production

As presented at Table 2, the cleavage rate was similar ($p > .05$) between all treatments. However, the blastocyst rates on D6 and D7 were higher in the control compared with the other groups, but similar ($p > .05$) between the other treatments. In addition, the BI rates at D6 were lower for embryos cultured in the presence of L-carnitine + CLA ($p < .05$) compared with the control group.

3.2 | Effect of delipidating substances, L-carnitine and trans-10 cis-12 CLA, on the lipid content of blastocysts in in vitro culture

To evaluate the effect of delipidating substances on lipid content, embryos were evaluated at D7 using Sudan Black staining. Embryos cultured in medium supplemented with L-carnitine presented lower amounts of lipids in the cytoplasm than the control ($p = .0138$) and CLA ($p = .0261$) groups (Figure 1). However, no difference ($p > .05$) in lipid content was found between the group supplemented with L-carnitine + CLA and the other treatment groups (Figure 1). Figure 2 shows images of developmental D7 expanded blastocysts

TABLE 2 Embryo development on D2, D6 and D7 of in vitro culture in the presence or absence (control) of delipidating agents, L-carnitine and/or trans-10, cis-12-CLA

		D2	D6				D7					
		Cleavage	Bi	Bl	Bx	Total	Bi	Bl	Bx	Bn	Be	Total
Treatments	No. of oocytes	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Control	616	583 (95)	52 (8)	46 (7)^{a}	18 (3)^{a}	116 (19)^{a}	40 (6)	83 (13)	175 (28)	1 (0)	2 (0)	301 (49)^{a}
L-carnitine	648	618 (95)	39 (6)	38 (6)^{ab}	7 (1)^{ab}	84 (13)^{b}	34 (5)	60 (9)	159 (25)	0	0	253 (39)^{b}
CLA	627	598 (95)	31 (5)	45 (7)^{ab}	12 (2)^{ab}	88 (14)^{b}	27 (4)	82 (13)	154 (25)	0	0	263 (42)^{b}
L-carnitine + CLA	597	556 (95)	29 (5)	4 (1)^{b}	4 (1)^{b}	75 (13)^{b}	31 (5)	72 (12)	127 (21)	0	0	230 (39)^{b}

Note: Data are presented by percentage. ^{a,b}Values with different letters in the same column differ significantly by the chi-square test ($p < .05$).
Abbreviations: Be, hatched blastocyst; Bi, initial blastocyst; Bl, blastocyst; Bn, hatching blastocyst; Bx, expanded blastocyst; CLA, trans-10 cis-12 isomer of conjugated linoleic acid.

(Bx) stained with Sudan Black B stain for lipid quantification analysis, 20X magnification optical microscope.

3.3 | Effect of delipidating substances, L-carnitine and trans-10 cis-12 CLA, during in vitro culture on the expression of genes related to bovine embryos

Gene expression analysis revealed similar ($p > .05$) transcript levels between all groups (Figure 3).

3.4 | Effect of delipidating substances, L-carnitine and trans-10 cis-12 CLA, during in vitro culture on embryo cryotolerance

To evaluate the response of embryos to cryopreservation, the rates of re-expansion and hatching were evaluated 24 and 48 hr after thawing (Table 3).

At 24 hr post-thawing, the re-expansion rate in the L-carnitine treatment group was lower than that in the control group ($p < .05$), whereas at 48 hr, the re-expansion rate was similar between treatments ($p > .05$). At 24 hr, the hatching rates were similar ($p > .05$) within the fresh and cryopreserved groups. When fresh and cryopreserved embryos were compared within each group, the hatching rate was only affected by cryopreservation in the L-carnitine and L-carnitine + CLA groups ($p < .05$) at that time point. At 48, the hatching rate in all groups was higher ($p < .05$) for fresh embryos compared with cryopreserved embryos; however, those cultured in the presence of L-carnitine presented lower hatching rates at 48 hr compared with the other cryopreserved groups ($p < .05$).

4 | DISCUSSION

In the present study, we evaluated the effects of L-carnitine and CLA on embryo culture. We hypothesized that the association of two delipidating agents during culture would decrease the amount of lipids and consequently improve the response to cryopreservation without affecting embryo development.

In the first experiment, the effects of L-carnitine and trans-10 cis-12 CLA on embryo production were evaluated. The results showed that cleavage was similar between the treatments. However, the presence of delipidants agents affected Bx development at D6 and D7, since embryos cultured in presence of those agents alone or in combination presented a lower Bx rate than the control group. Regarding cleavage rate, our results were similar to those observed previously using L-carnitine during the maturation and/or in vitro culture of bovine embryos (Chankitisakul et al., 2013; Phongnimitr et al., 2013; Takahashi et al., 2013). Similarly, a lack of effect on Bx rate when cultured in the presence of L-carnitine has also been reported (Chankitisakul et al., 2013; Held-Hoelker et al., 2017; Phongnimitr et al., 2013). As demonstrated for carnitine, the results obtained using CLA have also been controversial, with some studies showing a beneficial effect (Ghanem et al., 2014; Takahashi et al., 2013) on the embryo and others not showing a beneficial effect (Batista et al., 2014; Leão et al., 2015; Stinshoff, Wilkening, Hanstedt, Bollwein, & Wrenzycki, 2014).

It is unclear why those agents caused a decrease in Bx production; however, we should consider that L-carnitine acts on lipid metabolism, and ATP production can generate ROS as by-product (Harvey, Kind, & Thompson, 2002; Sutton-Mcdowall et al., 2012). Therefore, it is possible that the putative increase in ROS production could induce cellular damage, such as DNA fragmentation and apoptosis, and consequently block embryo development (Abdelrazik et al., 2009). Conversely, CLA is a polyunsaturated FA, which can redirect the embryo metabolic pathway to produce ATP and stimulate FA synthesis by glycolysis. This stimulation may favour the formation of intracytoplasmic lipids together with an imbalance in the reduction–oxidation state of the embryos, leading to mitochondrial disturbance and lipid accumulation (Barcelo-Fimbres & Seidel, 2007; De La Torre-Sanchez, Gardner, Prieis, Gibbons, & Seidel, 2006) compromising embryo development (Al Darwich et al., 2010; Marei, Wathes, & Fouladi-Nashta, 2009; Van Hoeck et al., 2013). This hypothesis is supported by our findings,

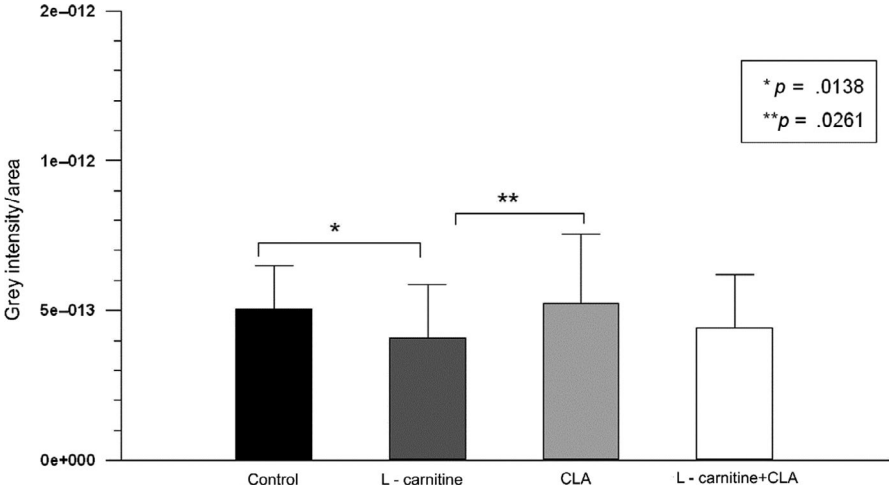

FIGURE 1 Accumulation of intracytoplasmic lipids in bovine embryos cultured in the presence of L-carnitine and the trans-10 cis-12 isomer of CLA. Values are expressed as grey intensity per area obtained by Sudan Black B staining in expanded blastocysts on D7. $p < .05$. CLA, conjugated linoleic acid

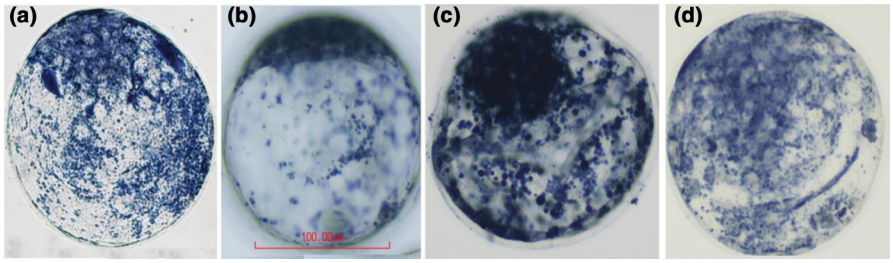

FIGURE 2 Images of expanded blastocysts (Bx) on D7 of development stained with Sudan Black B dye for analysis of lipid quantification. (a) Bx of the control group; (b) Bx culturedwith L-carnitine; (c) Bx cultured with CLA; and (d) Bx culture with L-carnitine and CLA. CLA, conjugated linoleic acid

whereby lipid content was evaluated by Sudan Black staining in embryos from the various treatment groups. In fact, contrary to expectations, embryos exposed to CLA did not present a decrease in lipid content, as demonstrated elsewhere (Absalón-Medina et al., 2014).

In this regard, the presence of L-carnitine during culture decreased the amount of lipids in the embryos, which was expected due to its effect on mitochondrial lipid metabolism, as previously observed (Ghanem et al., 2014; Takahashi et al., 2013).

In experiment 3, we evaluated the expression of genes important for embryo development and survival. The evaluated genes were previously reported to be related to lipid storage (PLIN2 and CPT2), apoptosis (BCL2L1, CASP3 and CASP8), the proliferation, differentiation and transformation of cells (FOSL1), thermal stress (HSPA5, HSPA1A and SOD2) and for repair of cellular damage (MSH6). Unexpectedly, no differences were observed in the transcription levels of these genes between embryos cultured in the presence or absence of L-carnitine and/or CLA. As noted, these substances act on lipid metabolism, and we hypothesized that they would induce changes in gene expression under the different treatments; however, no differences were found.

Finally, to confirm, whether culture in the presence of delipidant agents would affect embryo cryoresistance, post-thaw survival was evaluated. The results showed that L-carnitine had a negative effect on embryo resistance, since re-expansion rates at 24 hr were lower than those in embryos of the other groups. These results contrast with those obtained by Phongnimitr et al. (2013) and Chankitisakul et al. (2013). However, the previous studies differed from ours; those authors used L-carnitine during IVM, and the embryos were vitrified and not slow frozen. Nevertheless, the lower survival rate after thawing observed in the L-carnitine group was unexpected, since this lipid content in that group was also reduced. Reduced lipid levels can be beneficial to cell cryoresistance. However, the extent to which lipids can be reduced without affecting embryo development is not yet known. Therefore, we can exclude the possibility that the reduced lipid content may have had a negative effect on embryo development.

Unlike L-carnitine, the use of CLA in culture had no negative effect on re-expansion and hatch rates 24 and 48h after thawing, although no improvement in embryo development was observed after thawing.

When we compare our results with the literature, it is clear that these delipidant substances have already reduced the amount of lipids in bovine embryos (Batista et al., 2014; Chankitisakul et al., 2013; Phongnimitr et al., 2013; Takahashi et al., 2013), but we must consider in different studies that the embryos were kept in different culture conditions, using FBS or BSA, for example (Held-Hoelker et al., 2017). However, it is not yet known how much lipid can be removed from these embryos so that it does not impair their embryonic development after thawing. Moreover, it is not known

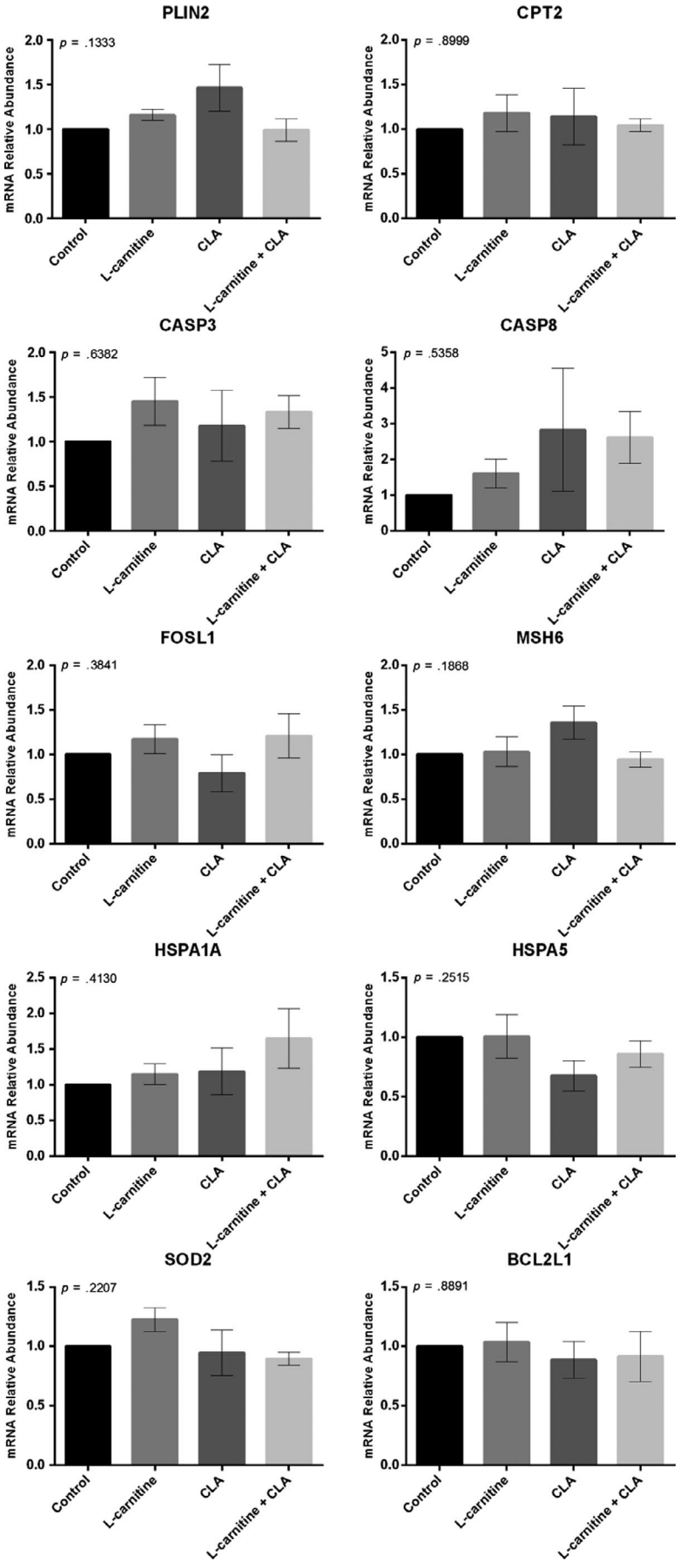

FIGURE 3 Relative abundance of mRNA encoding *PLIN2*, *CASP8*, *SOD2*, *CASP3*, *FOSL1*, *HSPA1A*, *CPT2*, *HSPA5*, *MSH6* and *BCL2L1* determined by quantitative polymerase chain reaction using expanded blastocysts cultured in vitro in the presence of L-carnitine and CLA. Mean ± standard error of the mean (*SEM*) of four biological replicates. The data (mean ± *SEM*) were normalized using the formula ΔΔCt as described by Pfaffl (2001), with GAPDH as an endogenous control. CLA, conjugated linoleic acid

TABLE 3 Re-expansion and hatching rates of frozen and fresh (control) Bx after culture in the absence (control) or presence of delipidating agents, L-carnitine and/or CLA

			Re-expansion rate		Hatching rate	
			24 hr	48 hr	24 hr	48 hr
Treatment		No. embryos	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Fresh	Control	41	–	–	15 (36.6)^{a,c,d}	30 (73.2)^{a}
	L-carnitine	58	–	–	26 (44.8)^{c}	46 (79.3)^{a}
	CLA	43	–	–	12 (27.9)^{a,b,c,d}	33 (76.7)^{a}
	L-carnitine + CLA	52	–	–	20 (35.8)^{a,c}	39 (75.0)^{a}
Frozen	Control	56	41 (73.2)^{a}	42 (75.0)^{a}	14 (25.0)^{a,e}	27 (48.2)^{b}
	L-carnitine	73	37 (50.7)^{b}	48 (65.8)^{a}	11 (15.1)^{b,e}	23 (31.5)^{c}
	CLA	65	42 (64.6)^{a,b}	43 (66.2)^{a}	14 (21.5)^{d,e}	36 (55.4)^{b}
	L-carnitine + CLA	75	45 (60.0)^{a,b}	59 (78.7)^{a}	12 (16.0)^{b,e}	39 (52.0)^{b}

Note: ^{a,b,c,d,e}Values with different letters in the same column differ significantly by the chi-square test ($p < .05$).
Abbreviations: Bx, expanded blastocysts; CLA, conjugated linoleic acid.

how much cultivation conditions may interact with these delipidant agents. Thus, it is clear that the concentration of the lipid substances used, the composition of the different culture media and the stage of IVP in which this supplementation was performed (Knitlova et al., 2017), besides the source of the oocytes and the species used (Lowe, Bartolac, Bathgate, & Grupen, 2017) may affect the results of embryonic production and lipid removal.

Based on these results, we conclude that the use of L-carnitine and/or CLA during in vitro culture was unable to improve the embryo response to cryopreservation when the slow-freezing method is used. Despite the decrease in the lipid content in the embryos exposed to L-carnitine, this raises a question about the changes that can be caused in the embryonic lipid metabolism. Furthermore, it is not yet known how much of the lipids can be removed from these embryos in a way that does not impair embryonic development after thawing or even affect the pregnancy rate when those embryos are transferred to recipients.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest of any kind.

AUTHOR CONTRIBUTIONS

Luzia Renata Oliveira Dias designed the study, analysed the data, drafted the paper and conducted the experiments. Ligiane Oliveira Leme and José Felipe Warmling Sprícigo designed the study, analysed the data and drafted the paper. Ivo Pivato and Margot Alves Nunes Dode designed the study and corrected the final manuscript of the article.

ORCID

Luzia Renata Oliveira Dias https://orcid.org/0000-0002-5066-2200

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